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# The LIM-homeodomain protein Isl-1 segregates with somatostatin but not with gastrin expression during differentiation of somatostatin/gastrin precursor cells

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**The gastric epithelium is renewed from stem cells in the isthmus of the gastric glands. We describe that the two neuroendocrine peptides gastrin and somatostatin are coexpressed by isthmic stem cells. Bromodeoxyuridine labeling indicates that the coexpressing cells divide and differentiate into gastrin and somatostatin cells, which remain in paracrine contact during most of their migration down into the gland. The coexpressing cells display nuclear immunoreactivity for the transcription factors Isl-1 and CREB, which have been implicated in somatostatin gene expression. Differentiated gastrin cells lack Isl-1 reactivity and show variable staining for CREB while differentiated somatostatin cells display Isl-1 and CREB reactivity.**

**Keywords:** somatostatin, gastrin, coexpression, Isl-1, homeobox, CREB

## Introduction

The gastric mucosa is renewed through divisions of stem cells present in the isthmus region (El-Alfy *et al.*, 1987; Lipkin, 1973). Recently, evidence was obtained suggesting that the regenerative units were monoclonal and originated from a single stem cell (Thompson *et al.*, 1990). Continued division, migration and differentiation of the offspring cells result in mature surface epithelial cells and in glandular exocrine and endocrine cells that express enzymes, mucus and hormones in a cell-specific manner. The mechanisms regulating cell-specific gene expression in the gastric mucosa are largely unknown. Several *cis*-regulatory sites necessary for expression of the gastric hormone gastrin have been identified (reviewed in Brand *et al.*, 1993), but, so far, no gastrin-transactivating factors have been cloned. The expression of the gastric neuroendocrine peptide somatostatin, which also is present in extragastric sites, has been shown to depend upon at least two different *cis*-regulatory promoter sequences for cell-specific expression. One of these is the cyclic AMP response element (CRE) that can interact with a CRE binding protein (CREB) (Montminy & Bilezikjian, 1987; Gonzalez *et al.*, 1989; for review see Meyer & Habener, 1993). A second site needed for islet cell expression can interact with the homeodomain LIM family protein Isl-1 (Leonard *et al.*, 1992), as well as with the homeodomain protein IPF-1 (STF-1/IDX-1; Leonard *et al.*, 1993; Ohlsson *et al.*, 1993; Milles *et al.*, 1994). Isl-1 was cloned as an insulin gene enhancer binding protein and subsequently shown to be present also in other tissues (Karlsson *et al.*, 1990; Thor *et al.*, 1991; Ericson *et al.*, 1992; Wang & Drucker, 1994).

Gastric somatostatin (D) cells and gastrin (G) cells often occur in close contact (Larsson *et al.*, 1979) and much data show that somatostatin regulates gastrin gene expression and gastrin secretion by a paracrine mechanism (Saffouri *et al.*, 1979, 1980; Chiba *et al.*, 1981; Wolfe *et al.*, 1983; Brand & Stone, 1988; Schubert *et al.*, 1988, 1991; Karnik & Wolfe, 1990; McIntosh *et al.*, 1991; Holst *et al.*, 1992). During studies on the development of such paracrine contacts we have observed that some cells of the isthmus region coexpress the gastrin and somatostatin genes (Larsson, 1994). We now present data showing that such G/D cells divide and differentiate into mature G and D cells that preserve contacts to each other during most of their migration into the gastric glands. This provided a unique model for studying how CREB and Isl-1 immunoreactivities segregate with specific gene activities during cellular differentiation.

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## Results

### *Distribution and development of gastrin-somatostatin (G/D) cells*

Sections of rat antropyloric mucosa were double-stained for gastrin and somatostatin immunofluorescence. Scattered few cells reacted for both peptides (G/D cells) while most immunopositive gland cells were reactive for either gastrin or somatostatin (Figure 1). The G/D cells were situated in the neck or isthmus region of the antropyloric glands while G and D cells typically occurred within the gland proper. As previously reported in a quantitative study (Larsson & Hougaard, 1994a) contacts between G and D cells were frequently observed in the body, but not at the base, of the glands. Similar stainings performed on juvenile (23 days old) rats revealed that the frequency of G/D cells was higher than in adult rats. A quantitative developmental study of G, D and G/D cells in neonatal rats was therefore performed. In close agreement with previously published biochemical and structural data (Larsson *et al.*, 1976; Larsson, 1977; Koshimizu, 1983; Zingg *et al.*, 1984; Okahata *et al.*, 1985; Brand & Fuller, 1988; Marino *et al.*, 1988) the frequencies of G and D cells increased in a characteristic pattern up to postnatal day 37 (p37) when their maximal frequency was attained, and thereafter decreased (Figure 2). G/D cells increased to a maximal frequency around p23 and then decreased in frequency by p37.

### *G/D cells contain both gastrin and somatostatin mRNA's*

*In situ* hybridizations for gastrin and somatostatin mRNA were performed using biotinylated probes on thin adjacent sections of rat antropyloric mucosa (p23). In addition, double *in situ* hybridizations using combined $^{35}$S-labeled somatostatin and biotin-labeled gastrin probes were performed. Both approaches documented that scattered few cells expressed both mRNA's while most reactive cells expressed only one mRNA species (Figure 1). The double *in situ* hybridizations were easiest to analyse. However, in contrast to the biotin-labeled probes, $^{35}$S-labeled probes also reacted with scattered cells in the lamina propria. This reaction was unspecific as it

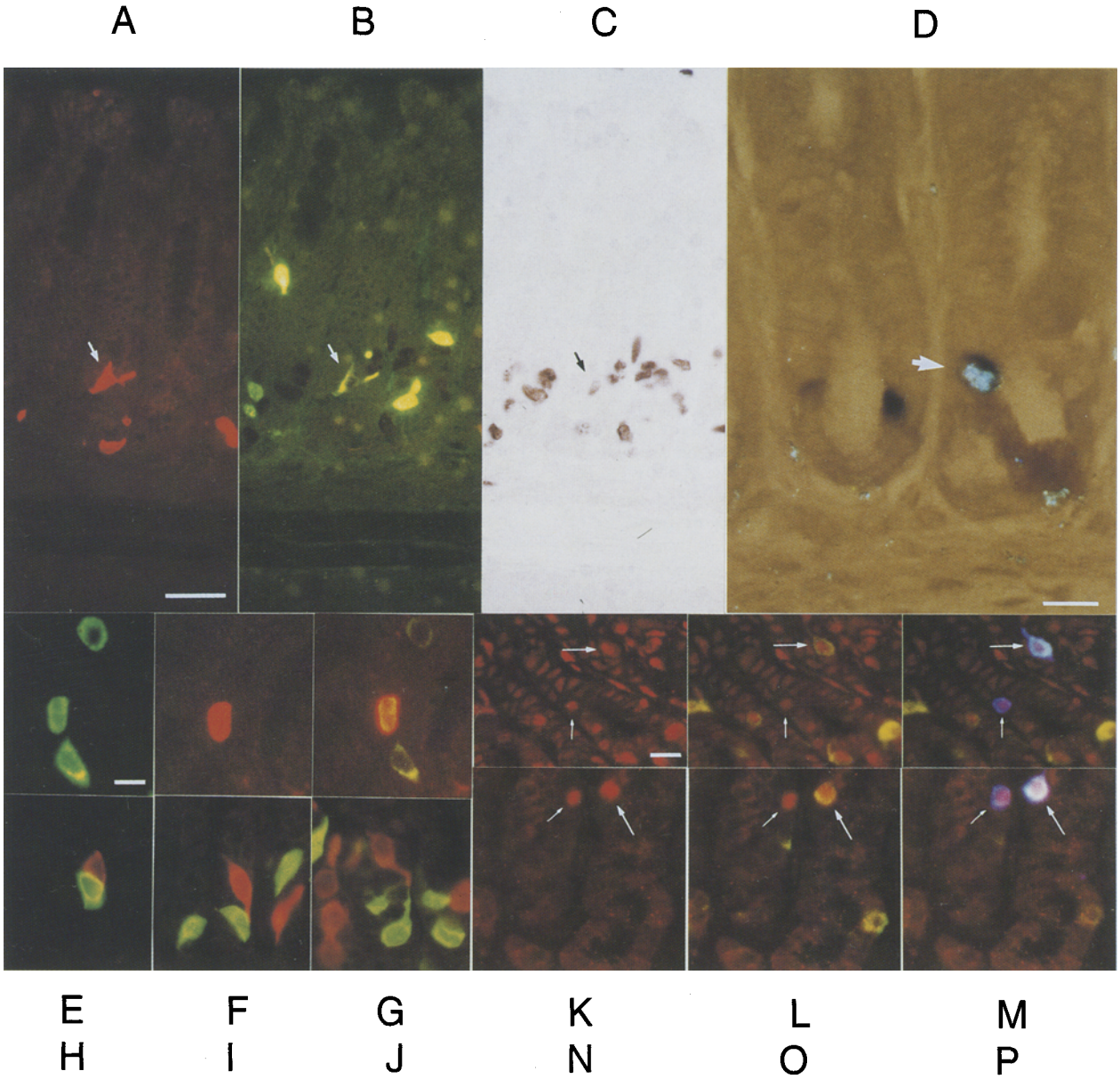


**Figure 1** (**a**–**c**) Sections through antropyloric mucosa of a 23 day old rat injected with BrdU 5 h before sacrifice. The section was triple-stained for detection of somatostatin (**a**, red immunofluorescence; scale bar 50 μm), gastrin (**b**, green immunofluorescence) and BrdU (**c**, brown immunoperoxidase reaction). Note that one cell (arrow) in the isthmus displays both gastrin, somatostatin and BrdU immunoreactivity; (**d**) Section through rat antropyloric mucosa (p23) double in situ hybridized for somatostatin mRNA by a $^{35}$S-labeled probe and gastrin mRNA by a biotinylated probe and examined in polarized epiillumination. One cell (arrow) displays both dark immunoalkaline phosphatase reaction product for gastrin mRNA and luminescent autoradiographic silver grains indicating somatostatin mRNA, while other cells are positive for gastrin mRNA only (scale bar 20 μm); (**e**–**j**) rat antropyloric mucosa double-stained for gastrin (green) and somatostatin (red) immunofluorescence. In (**e**–**j**) is shown an anaphase-like figure in a G/D cell using single green (**e**), single red (**f**), and double exposure (**g**) while (**h**–**j**) depicts double exposures of different types of contacts seen between G and D cells (scale bar 20 μm). (**k**–**m**) Immunofluorescence triple staining for CREB (red), gastrin (green) and somatostatin (blue) in antropyloric mucosa of p27 rat, photographed using single red (**k**) and double red/green (l) and triple exposure (**m**). Note presence of CREB staining in all cells including a cell coexpressing gastrin and somatostatin (long arrow) and in differentiated somatostatin (short arrow) and gastrin cells (scale bar 40 μm). (**n**–**p**) Triple immunofluorescence for Isl-1 (red), gastrin (green) and somatostatin (blue) photographed as in (**k**–**m**). Note nuclear stain for Isl-1 in cells coexpressing gastrin and somatostatin (long arrow) as well as in somatostatin cells (short arrow), but lack of detectable Isl-1 in gastrin cells

was obtained also with a $^{35}$S-labeled probe specific for human insulin and was also seen after RNase pretreatment. No epithelial cells were, however, reactive in the controls. It has previously been reported that unspecific lamina propria staining may be obtained by other $^{35}$S-labeled probes (Panula & Wasowicz, 1993). In practice, the unspecific labeling caused no interpretative problems as it was anatomically distinct from the specific epithelial labeling. Moreover, all controls were negative with the biotin-labeled probes and use of these on adjacent sections further attested coexpression of both mRNA's. Further controls consisted of Northern blots which showed that the gastrin and somatostatin probes reacted only with the appropriately sized bands of total rat antropyloric RNA.

### *Evidence for DNA-synthesis and mitoses in G/D cells*

Twelve rats were injected with BrdU on p25 and sacrificed 2, 5 and 26 h later. Sections of antropyloric mucosa were triple immunostained for BrdU, gastrin and somatostatin and the percent immunoreactive cells with BrdU-labeled nuclei determined. By 5 h after injection the labeling index was highest

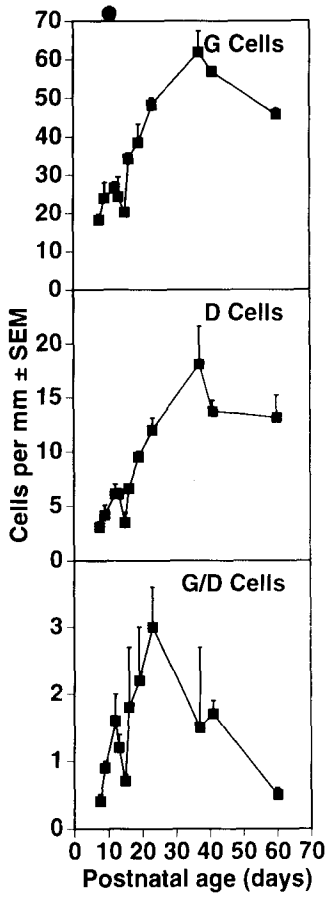


**Figure 2** Development of G, D and G/D cells in neonatal rats. Note that the frequency of G/D cells peaks at p23 while that of G and D cells peaks at p37. Results are expressed as cell numbers per mm ± standard error of the mean (SEM) and are based on 2–4 rats for each point

for G/D cells, lower for G cells and lowest for D cells. By 26 h, there was a significant decrease in labeling index for G/D cells ($P<0.05$) while that of D and G cells continued to increase (Figure 3). Although the isthmus represents the stem cell zone of the gastric glands, BrdU-labeling was also detected deeper down in the gland proper (Figure 4). These data indicate that both G, D and G/D cells synthesize DNA and divide. Mitotic-like figures were also recognized in G/D cells (Figure 1).

*Correlation of transactivating factor immunoreativities to gastrin and somatostatin gene expression*

Antropyloric mucosa from juvenile (p23–p27) rats were stained with polyclonal CREB antiserum. Virtually all nuclei of both epithelial and subepithelial cells showed variable intensities of staining. In addition, few scattered epithelial cells showed variable cytoplasmic staining. Triple stainings for CREB, gastrin and somatostatin revealed intense reactivity for CREB in the nuclei of G/D cells and D cells (Figure 1). G cells typically showed both nuclear and cytoplasmic reactivity for CREB, but occasional G cells displayed only cytoplasmic CREB staining. The monoclonal Isl-1 antibody reacted with few scattered nuclei of epithelial cells. Triple staining revealed that the G/D cells and D cells were Isl-1 positive while G cells consistently failed to stain for Isl-1 (Figure 1). Additionally, very few, unidentified, cells of the gastric mucosa showed nuclear Isl-1 staining. Stainings for CREB and Isl-1 were also performed in adult (p50–60) rats. This produced the same general pattern as described above. However, although strong nuclear Isl-1 immunoreactivity was detected in adult G/D cells the degree of staining of

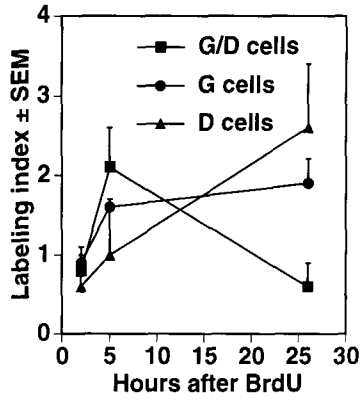


**Figure 3** Labeling indices for G, D and G/D cells obtained 2, 5 and 26 h after a single BrdU injection. Sections triple immunostained for BrdU, gastrin and somatostatin were used (cf. Figure 1a–c). The percentage of BrdU-labeled cells was determined in four rats for each time (± SEM). Total numbers of cells counted were 2 h (G:1019; D:335; G/D:308), 5 h (G:1447; D:580; G/D: 480) and 26 h (G: 1554; D:530; G/D: 284). The decrease in labeling index for G/D cells between 5 and 26 h is significant. (Mann-Whitney U-test: $P<0.05$) as is the increase in labeling for D cells between 2 and 26 h ($P<0.05$) and for G cells between 2 and 5 h ($P<0.05$)

differentiated D cells was variable, ranging from negative to strongly positive. As in young rats G cells were invariably negative for Isl-1.

## Discussion

Cells immunoreactive for gastrin and somatostatin were found in the isthmus zone of adult rat antropyloric mucosa. In addition, higher frequencies of such cells were detected in younger rats. Their frequencies in young rats could be correlated to the development of mature G and D cells and showed a maximum around postnatal day 23. Somewhat later, maximal development of mature G and D cells was attained. Double *in situ* hybridizations proved the presence of both gastrin and somatostatin mRNA in G/D cells. Since G/D cells were most numerous in juvenile rats these rats were used for some of the quantitative experiments, including BrdU-labeling.

The occurrence of G/D cells in the regenerative (isthmus) region of the gastric glands as well as their ontogenetical development and BrdU-labeling kinetics suggest that they represent precursors to G and D cells. We hypothesize that stem cells of the isthmus divide and that part of their daughter cells differentiate into G/D cells. As shown by the BrdU-labeling pattern the G/D cells synthesize DNA. The need to perform double-staining immunofluorescence and, above all, the paucity of G/D cells made identification of classical metaphases difficult. Nevertheless, cell figures suggestive of anaphases/telophases were observed. Together with the rapid turnover of the cells, as shown by BrdU-labeling, this strongly suggests that the G/D cells actively divide. We postulate that the resulting daughter cells differentiate into separate G and D cells that remain in funtional (paracrine) contant during most of their migration down the gland (Figure 5; cf. Figure 1h–j). This model explains how paracrine cell contacts can arise. Eventually, due to continued cell division, the contacts are lost leading to separation of G and D cells at the base of the glands. Our hypothesis unifies previous data suggesting an origin of G cells from either the isthmus (Fujimoto *et al.*, 1980; Shimoda *et al.*, 1990) or from division of mature G cells (Lehy & Willems, 1976; Lehy, 1982). Mitoses of mature G and D cells have been observed in the gland proper (Lehy, 1982) and different mitotic rates may cause the numerical inequality between G and D cells.

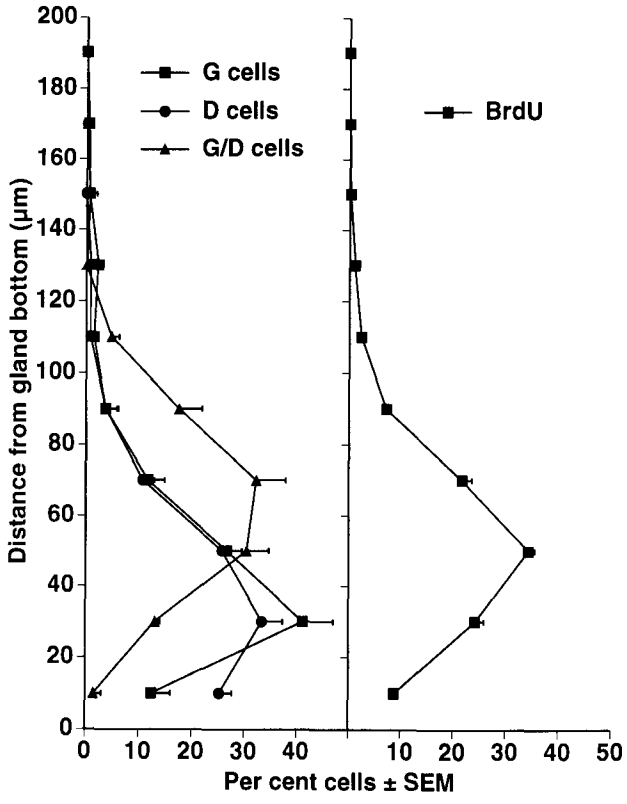


**Figure 4** Distributions of G, D and G/D cells (left) and BrdU-labeled nuclei (right) within gastric glands of p25 rats. The distances from the base of the gland to the center of the nucleus was measured in perfectly transverse sections. Note that G/D cells ($n = 117$) occur higher up in the glands than do G ($n = 351$) and D cells ($n = 236$). Also note that 5 h after injection BrdU-labeled nuclei ($n = 2018$) occur both in the isthmus region (40–90 μm) as well as in the gland proper. The data were derived from measurements on two specimens each for three rats and are represented as means ± SEM

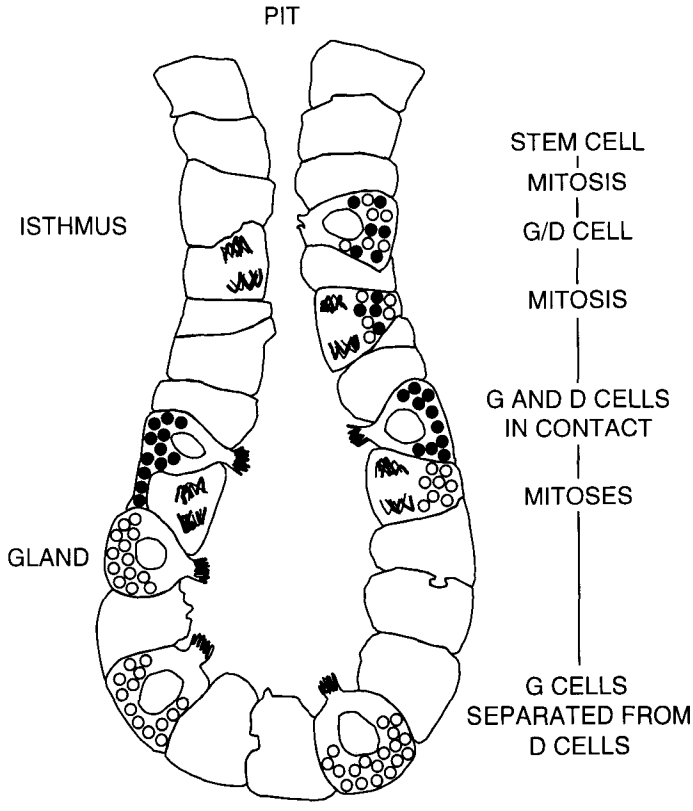


**Figure 5** Drawing illustrating our concept of G and D cell differentiation and migration in antropyloric glands

All of our data fit with the hypothesis that G/D cells constitute precursors to G and D cells. An alternative interpretation would be that the G/D cells constitute examples of errors of differentiation. If this were the case such errors would be very common indeed and would probably necessitate effective elimination mechanisms. In contrast, our results suggest that G/D cells actively synthesize DNA and divide. The gastrin antisera employed have been found to react with biologically active amidated gastrin forms (Larsson & Rehfeld, 1979) and do not detect unprocessed gastrin precursors (Larsson, unpublished data). It is therefore likely that at least some processing of the gastrin precursor takes place in the G/D cells. If G/D cells represented an error of differentiation this error must also induce processing of the gastrin precursor. On the balance we therefore propose that G/D cells represent true precursors to G and D cells. Since these cells simultaneously express both the gastrin and somatostatin genes they constitute a valuable model for examining how transcription factor immunoreactivities correlate to gene expression. Such correlations represent a necessary first step towards more experimental studies on the direct relation between specific transcription factors and gene expression *in vivo*.

The Isl-1 protein belongs to the LIM-family of homeodomain transcription factors. The LIM-domain is a $Zn^{2+}$-binding domain originally recognized by homology between the homeodomain proteins lin-11, Isl-1 and mec-3 (Way & Chalfie, 1988; Freyd *et al.*, 1990; Karlsson *et al.*, 1990). Lin-11 and mec-3 are *C. elegans* proteins necessary for asymmetrical cell divisions involved in the formation of vulval cells and touch-receptor neurons, respectively (Freyd *et al.*, 1990; Way & Chalfie, 1988). Mec-3 is also required for expression of the touch-cell genes mec-4 and mec-7 (Xue *et al.*, 1993; and references therein). In juvenile rats all cells that expressed somatostatin also showed nuclear staining for Isl-1 and CREB while cells that only expressed gastrin lacked staining for Isl-1. This observation indicates that cells committed to the gastrin lineage down-regulates Isl-1. The concordant presence of somatostatin, Isl-1 and CREB immunoreactivities in D cells of juvenile rats are consistent with findings in islet cells that Isl-1 and CREB together promote somatostatin gene expression (Leonard *et al.*, 1992). Thus, these factors may also be important for regulating gastric somatostatin gene expression. While all somatostatin cells in juvenile rats also showed nuclear staining for Isl-1, somatostatin cells of adult rats showed a more variable staining. Whether this variable staining reflects variable degrees of somatostatin gene activity is presently unknown. By analogy to the *C. elegans* homeodomain proteins lin-11 and mec-3, additional or alternative roles for Isl-1 in G and D cell development could be considered. One possibility is that Isl-1 would be needed for the asymmetrical cell divisions of G/D cells that, according to our hypothesis, result in separate G and D cells. In addition, down-regulation of Isl-1 in maturing G cells could be important for down-regulating somatostatin gene expression in these cells.

Several *cis*-regulatory sites and potential transactivating proteins have been implicated in regulating the gastrin gene but, so far, none have been cloned (reviewed in Brand *et al.*, 1993). Such elements include sequences that mediate epidermal growth factor stimulation and somatostatin inhibition of the gene. The latter site encompasses an E box motif. In addition, a GAGA-repeat sequence downstream of the TATA box and a unique sequence mediating both positive and negative regulation of the gastrin gene have been detected (Brand *et al.*, 1993; Tillotson *et al.*, 1994). Inspection of 5′-flanking sequences of the rat and human gastrin gene also reveals presumptive Isl-1 binding sites (unpublished data). The Isl-1 binding sites occur much upstream of the human gene (800 bases and more) but are present already in the about 200 bases of 5′-flanking DNA of the rat gene that

so far has been sequenced (Brand *et al.*, 1993). This is interesting as only human gastrin transgenes with very long 5′-flanking sequences are expressed in antrum of transgenic mice, while a rat gastrin transgene with only 450 base pairs of 5′-flanking DNA is expressed (Brand *et al.*, 1993; Montag *et al.*, 1993). The fact that gastrin cells down-regulate Isl-1 suggests that it does not play a major positive role for gastrin gene expression. Its role, if any, for gastrin expression remains unclear. Other transactivating factors, however, like IPF-1 (STF-1/IDX-1; Leonard *et al.*, 1993; Ohlson *et al.*, 1993; Miller *et al.*, 1994) can bind to the same motif as Isl-1. Accordingly, yet unknown transactivating factors of this type could afford a positive regulation of gastrin gene expression and compete with Isl-1 for binding to the same *cis*-regulatory sites.

## Materials and methods

### *Tissue material*

Rats of either sex were sacrificed by carbon dioxide asphyxia when 1, 3, 4, 6, 7, 8, 9, 12, 13, 15, 16, 19, 23, 25, 27, 37, 41, 50 and 60 days old. The antropyloric region of the stomach, represented by a strip 1 mm wide on the major curvature from the pylorus and 1.5 mm up in the oral direction was dissected out. Twelve 25 days old rats were intraperitoneally injected with 3 mg per 100 g body weight of bromodeoxyuridine (BrdU, Amersham International, Buckinghamshire, England) and sacrificed after 2, 5 and 26 h. Tissues were fixed in 10% neutral buffered formalin and embedded in paraffin or were freeze-dried, paraformaldehyde vapour-fixed and paraffin-embedded (Larsson, 1988). In addition, 23, 25, 27, 50 and 60 days old rats were perfusion-fixed with freshly prepared 4% paraformaldehyde in 0.1 M sodium phosphate buffer. Antropyloric strips were dissected out, post-fixed overnight and prepared for cryostat sectioning (Larsson, 1988).

### *Immunocytochemistry*

5 μm paraffin sections were dewaxed, hydrated and stained with polyclonal rabbit (No 2717) or guinea pig (GPL 22) antibodies to synthetic human gastrin I or with polyclonal rabbit (R213/3) or monoclonal mouse antibodies (SOM 018; Novo Nordisk, NovoClone, Bagsværd, Denmark) to synthetic somatostatin-14 (Larsson & Rehfeld, 1979; Larsson *et al.*, 1979; Larsson & Hougaard, 1994b). In addition, cryostat sections were stained with a monoclonal antibody to Isl-1 (No 40-2D6, Developmental Studies Hybridoma Bank, Dept. Biology, University of Iowa, Iowa) and a rabbit polyclonal antiserum to recombinant CREB (No 06-244, Upstate Biotechnology Inc., Lake Placid, NY) (Ginty *et al.*, 1993). Double and triple immunofluorescence staining was carried out using combinations of primary antibodies from different species with species-specific secondary antibodies conjugated to fluorescein isothiocyanate (FITC), tetramethylrhodamine isothiocyanate (TRITC) or 7-amino-4-methyl-coumarine-3-acetic acid (AMCA) (Dakopatts A/S, Glostrup, Denmark and Accurate Chemical & Scientific Cooperation, Westbury, NY) yielding green, red and blue fluorescence, respectively. Controls included conventional staining controls, type-matched antibodies and absorptions against synthetic human gastrin I and somatostatin-14 (Peninsula Laboratories, Merseyside, England) and were uniformly negative. Sections from BrdU-injected animals were first pretreated with 0.015% pepsin in 0.2 M HCl for 20 min and then stained for BrdU using a monoclonal mouse BrdU-DNase solution (cell proliferation kit, RPN 20, Amersham) and detection by peroxidase-labeled antimouse IgG2a and diaminobenzidine development. Subsequently, the BrdU-stained sections were restained for double immunofluorescence detection of gastrin and somatostatin immunoreactants, as described above. Sections were examined in a Leica Aristoplan microscope equipped for epiillumination using prealigned selective FITC, TRITC and AMCA filter cubes and photographed using double and triple exposures. Cell frequencies were determined by counting the number of immunoreactive cells per mm length of mucosa from the pylorus and 1.5 mm upwards on the major curvature in 2–4 rats of each age. The distribution of cells along the length of the gland was obtained by measuring the distance from the base of the gland to the center of the nucleus of immunoreactive cells using a calibrated eyepiece graticule. All results were expressed as means ± standard error of the mean (SEM). For statistics, the Mann-Whitney *U*-test was used.

### In situ *hybridization*

Biotinylated oligodeoxynucleotides complementary to mRNA coding for (1) rat gastrin (RatGAS4 position 212-238; EMBL ID: RNPPG34 Rel 25; Fuller *et al.*, 1987); (2) human gastrin (HumGAS position 6641-6664; EMBL ID: HSGASTA Rel 31; Kariya *et al.*, 1986); and (3) rat somatostatin-14 (RatSRIF position 367-393; EmBL, ID: RNSOMA, Rel. 3; Goodman *et al.*, 1982) were synthesized as described (Larsson & Hougaard, 1992, 1993a, 1994a). Dewaxed and hydrated sections were submitted to an *in situ* hybridization protocol published elsewhere (Larsson & Hougaard, 1993b, 1994a). Hybridizations were performed at 15°C below the melting point (Tm) of the hybrids and stringency washes were at 10°C below the Tm. Detection was by a monoclonal antibiotin-streptavidin-alkaline phosphatase procedure (Larsson & Hougaard, 1994a). In addition, a nonbiotinylated RatSRIF oligodeoxynucleotide and a control oligodeoxynucleotide (complementary to human insulin mRNA) were tailed with [$^{35}$S]-dATP (Amersham; specific activity 1 mCi/nmol) using terminal deoxynucleotidyl transferase (Enzo Diagnostics Inc., New York, NY) and purified by repeated ethanol precipitation. Hybridizations were carried out with these probes alone or combined with biotinylated RatGAS4 probe followed by immunocytochemical and autoradiographic detection procedures (Miller *et al.*, 1993; Larsson & Hougaard, 1994b). Controls consisted of hybridizations 20°C above the Tm, use of RNase-predigested sections and of mismatching species-specific (human/rat) probes. In addition, total rat antropyloric RNA was extracted, subjected to denaturing formaldehyde electrophoresis, as described (Auffray & Rougeon, 1980) and transferred to nitrocellulose membranes (Millipore type HA, 0.45 μm; Waters-Millipore Corporation, Milford, Massachusetts, USA) by capillary blotting. Membranes were hybridized using biotinylated probes to rat gastrin and somatostatin, blocked for 40 min in 0.2% Tween 20 in 2 × SSC and submitted to the ASAP detection method, as described above.

### Note added in proof

After submission of this manuscript a paper appeared (Tsuchida, T., Ensini, M., Morton, S.B., Baldassare, M., Edlund, T., Jessell, T.M. & Pfaff, S.L. (1994). *Cell*, **79**, 957–970) reporting on the existence of a chicken gene product, Isl-2, that is closely homologous to Isl-1. The authors found that most available Isl-1 antibodies also reacted with Isl-2. We have repeated our experiments using mild postfixation of unfixed cryostat sections. Although this procedure is suboptimal for gastrin immunocytochemistry we were able to reproduce our findings reported in the present paper. In addition, the weaker fixation conditions permitted the visualization of Isl-1-like immunoreactivity in a number of mesenchymal cell nuclei. This could reflect lower concentrations of Isl-1 in mesenchymal nuclei. Alternatively, if Isl-2

exists in the rat, this molecule could contribute to the staining. In the light of this new information definite distinction between Isl-1 and Isl-2 must await specific antibodies or hybridization probes.

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