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Clonally Related Cells Are Restricted to Organ Boundaries Early in the Development of the Chicken Gut to Form Compartment Boundaries

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The gut organs are all derived from a simple, undifferentiated, linear gut tube. We analyzed the lineage relationships of cells derived from this gut tube in chicken embryos, determining where the progeny of a single cell are located within the gut. We find that daughter cells derived from a single progenitor can populate both the gizzard (chicken stomach) and the small intestine early in development, but that clonally related cells are restricted to a single organ by stage 12. We also find that clonally related cells can populate different mesodermal layers within the radial axis of the gut throughout all of the stages tested in these experiments. Many genes that have organ-specific expression patterns within the gut have been isolated. The onset of these restricted expression patterns correlates with the time that clonal boundaries appear to form, suggesting that these genes might be involved in the establishment of compartment boundaries, which prevent cells on one side of the boundary from intermingling with cells on the other side of the boundary. © 2000 Academic Press

Key Words: **retrovirus; lineage; embryo; chick; gut; compartment boundaries.**

INTRODUCTION

The gastrointestinal tract is critical for the survival of an organism, being responsible for the intake of food, the digestion of ingested food, the absorption of nutrients, and the expulsion of waste products from the body. These functions are carried out by a series of specialized organs, each with a unique epithelial and mesenchymal architecture. During embryogenesis, the vertebrate gut begins as a simple tubular structure with no morphological distinction between organs. Organ primordia are established within the gut tube in a characteristic anterior–posterior pattern. The organ primordia then differentiate into structures specialized for their distinct physiological roles in digestion. For example, the stomach is composed of an epithelial layer, derived from visceral endoderm, specialized for secretion of hydrochloric acid, pepsinogen, and mucus. The stomach epithelium is surrounded by a thick mesodermally derived tissue, which consists of a layer of undifferentiated connective tissue and three layers of smooth muscle. In contrast, the pancreas, which forms as an outgrowth from an adjacent portion of the gut tube, has a complex, specialized secretory epithelium composed of both endocrine and exocrine tissue with little surrounding mesoderm.

The undifferentiated gut tube begins as a flat sheet of endoderm that is subsequently "rolled" into a tube (Grapin-Botton and Melton, 2000). The endoderm sends a signal to recruit the overlying mesoderm to become gut mesoderm (Apelqvist *et al.*, 1997). This "rolling" begins at both the anterior (anterior intestinal portal/AIP) and the posterior (caudal intestinal portal/CIP) regions of the embryo. The flat sheet of endoderm is then zippered closed from both the AIP and the CIP until these two forming tubes meet in the middle of the embryo to form the primitive gut tube. Once the gut tube is formed, organ differentiation occurs. The phenotypes of the mesoderm and endoderm of the organs of the gut are regulated by a complex set of epithelial–mesenchymal signals (Kedinger *et al.*, 1986, 1990; Montgomery *et al.*, 1999). For instance, endodermal organ-specific differentiation is regulated by signals from the overlying mesodermal tissue (Kedinger *et al.*, 1986; Haffen *et al.*, 1989; Apelqvist *et al.*, 1997; Roberts *et al.*, 1995, 1998).

Some of the molecules that are involved in epithelial–mesenchymal signaling in the gut have recently been elucidated. Hox genes, encoding a family of homeobox-containing transcription factors, are regionally expressed in overlapping anterior–posterior domains within the gut tube (Roberts *et al.*, 1995; Yokouchi *et al.*, 1995). The posteriorly expressed Hox genes are expressed in the gut mesoderm,

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with the exception of *hoxd13* and *hoxa13*, which are also found in large intestinal endoderm (Roberts *et al.*, 1995). This regionalization of gene expression patterns matches the organ boundaries, even before distinct morphological organ boundaries are present. Evidence for functional roles for these genes in gut morphogenesis has been obtained from the analysis of loss-of-function mutants, such as mice carrying deletions in the posteriorly and mesodermally expressed *hoxd13* and *hoxa13* genes, which display anterior transformations in the mesoderm of the hindgut and anal sphincter (Kondo *et al.*, 1996). Further, misexpression of HoxD13, which is normally restricted to the posterior large intestinal mesoderm, throughout the mesoderm of the small intestine results in a partial transformation of the small intestinal endoderm into a large intestinal phenotype (Roberts *et al.*, 1998). These experiments suggest that Hox genes play a role in regionalization of the gut and in relaying a signal from the overlying mesoderm to the underlying endoderm, which results in patterning the endoderm.

The expression of posterior Hox genes is initiated by the secreted molecule Sonic Hedgehog (Shh) (Roberts *et al.*, 1995). Shh is expressed throughout the endoderm of the developing gut and is a critical signal in patterning the overlying mesoderm (Apelqvist *et al.*, 1997; Roberts *et al.*, 1998). Shh activates the expression of Hox genes at the CIP in a temporal order, coinciding with their colinear position in the chromosome. For instance, HoxD10 is activated before HoxD11 in the gut mesoderm, which results in HoxD10 having an anterior border of expression that is more rostral than the anterior border of expression of HoxD11. Although Shh is expressed throughout the gut endoderm, the Hox genes are activated in organ-restricted regions within the mesoderm. These results suggest that either the mesoderm is prepatterned before it sees the Shh signal or the mesoderm is patterned as it is exposed to Shh signaling in temporal order; starting at the CIP. In addition, some prepatterning of the endoderm has been suggested to exist, based upon the organ-restricted expression pattern of the transcription factors *CdxA*, *Sox2*, and *Pdx1* (Ishii *et al.*, 1997, 1998; Hebrok *et al.*, 1998). The expression pattern of *CdxA* is restricted to the small intestinal endoderm and its expression is under the control of the overlying mesoderm (Ishii *et al.*, 1997). *Sox2* is expressed only in the stomach endoderm and its expression is also modulated by the overlying mesoderm (Ishii *et al.*, 1998). *Pdx1* expression is found in the duodenal and pancreatic endoderm and plays a role in pancreatic outgrowth and differentiation (Offield *et al.*, 1996; Hebrok *et al.*, 1998).

Although the morphogenesis of the gut tube from the AIP and CIP involves cell movements, it is unclear what types of individual cell migrations occur in the early gut. Many fate mapping methods track populations of cells, but cannot resolve individual daughter cells derived from a single progenitor cell (Dymecki, 1996; Dymecki and Tomasiewiech, 1998; Nomura *et al.*, 1998). For example, site-directed recombinases such as FLP or CRE can be used to define cell fates in the mouse, but lack single-cell resolution (Dymecki, 1996; Dymecki and Tomasiewiech, 1998). Similarly, population level fate mapping can be carried out in chicken embryos by application of lipophilic dyes. For example, fate mapping of the chicken gut has been performed using the fluorescent marker DiI to label groups of endodermal and mesodermal cells at stages 8–11 (Hamburger and Hamilton, 1951; Matsushita, 1995, 1996, 1999). This labeling experiment found that the final fate of labeled mesodermal cells corresponds to the initial anterior–posterior position of the labeled patch of cells, such that anteriorly labeled cell patches labeled anterior organs, although there was not a sharp border between cells fated to a particular organ (Matsushita, 1995). However, since large numbers of cells are labeled at a time using this approach, it is impossible to evaluate whether any migration of cells between organs had taken place.

In addition to the anterior–posterior regionalization of the gut, there exists a radial organization of the mesoderm of the gut (Figs. 1A and 1B). The mesoderm of the gastrointestinal tract is arranged into three concentric layers around the underlying endoderm, marked by Shh expression (Roberts *et al.*, 1995). The innermost region of the mesoderm is the submucosal layer, located adjacent to the endoderm, which is a region of undifferentiated connective tissue, marked by BMP-4 expression (Sukegawa *et al.*, 2000). The next layer is the muscularis layer, which is composed of layers of smooth muscle, marked by FKBP/SMAP expression (Fukuda *et al.*, 1998). The outermost mesodermal layer is that of the serosa, which is a thin layer of epithelium that surrounds all of the organs of the gut. While Shh has been implicated in patterning some aspects of the radial axis of the gut (Sukegawa *et al.*, 2000), it is not known how the progenitor cells that give rise to the radial mesodermal layers of the gut organs are related. For example, can clonally related cells derived from a single progenitor span the various mesodermal layers of the gut, such as the submucosa and muscularis layers?

We have addressed these cell lineage and migration questions by the utilization of the CHAPOL retroviral system, which allows one to identify daughter cells derived from a single progenitor (Golden *et al.*, 1995). This system utilizes a replication-incompetent retrovirus that contains the retroviral *gag* and *pol* genes, the placental *alkaline phosphatase* gene, and a 24-base degenerate oligonucleotide insert (Golden *et al.*, 1995; Lin and Cepko, 1999). The retrovirus is injected into chicken embryos and the embryos are harvested at desired time points and then stained for alkaline phosphatase (AP) activity, which identifies all cells infected with the retrovirus. The AP^+ tissue is embedded in mounting medium and sectioned using a microtome, the AP^+ cells are individually picked from the sectioned tissue, and PCR is performed to amplify the degenerate oligonucleotide using specific primers (Figs. 1C and 1D). The PCR product is sequenced and comparisons of sequences are then made to determine sister cells (Fig. 1D) (Golden *et al.*, 1995; Lin and Cepko, 1999). We have applied this technique to the gut (Fig. 2) to answer two questions.

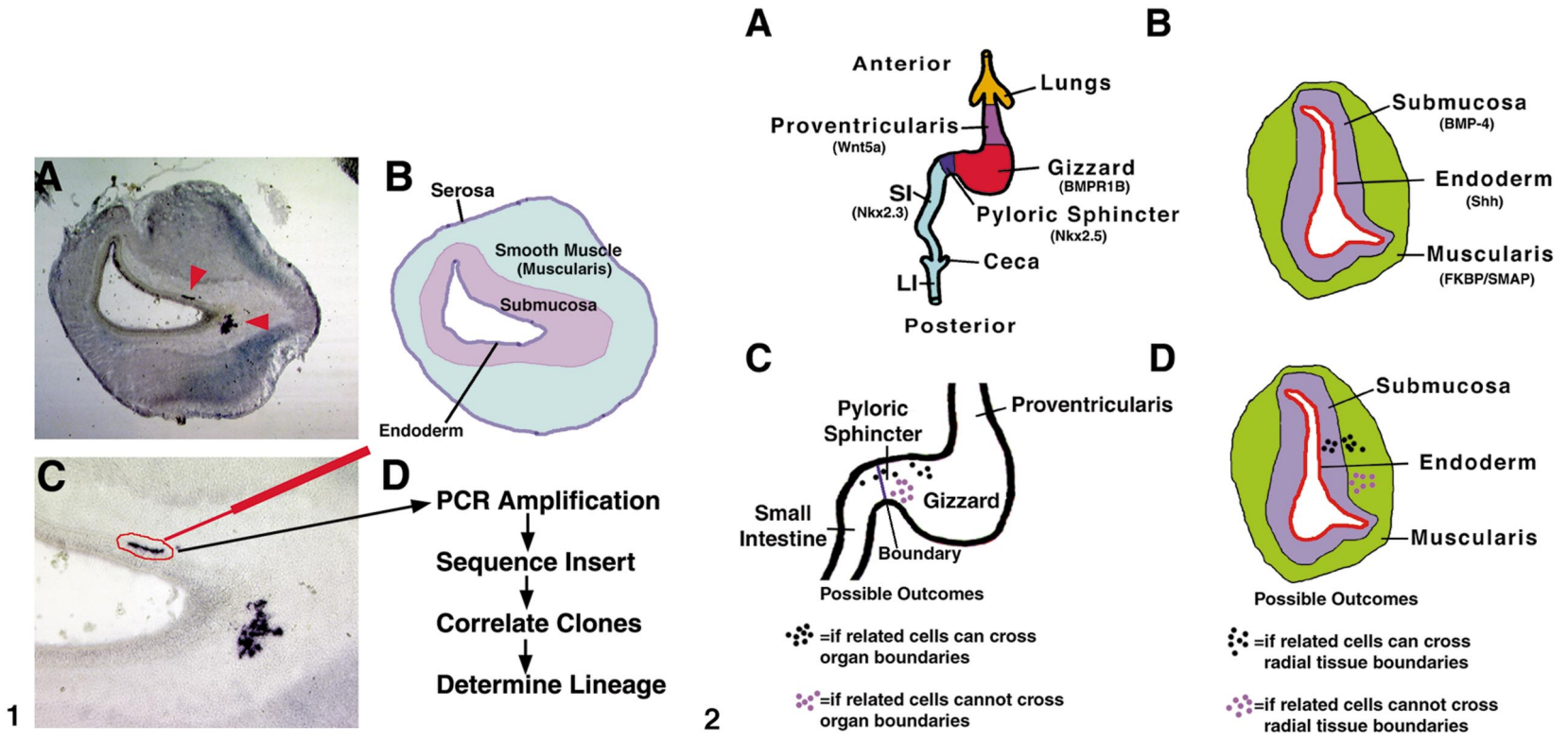
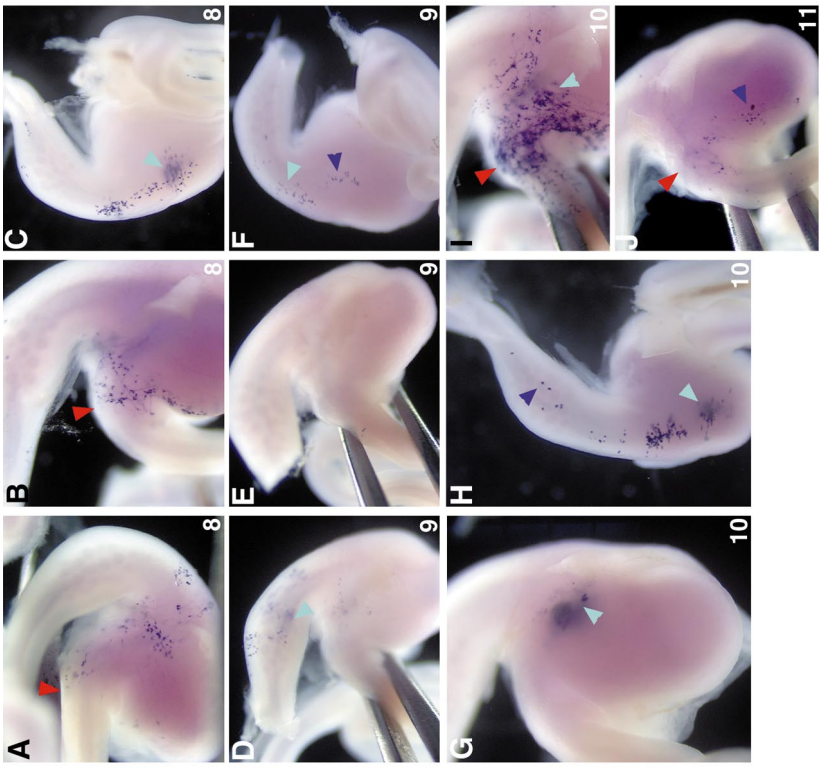


FIG. 1. Schematic example of the protocol used in this study. (A) Cross section through a gizzard that has been stained for alkaline phosphatase (AP) activity showing staining of two cell clusters (red arrowheads). (B) Schematic cross section through a gizzard showing the layers of the gizzard and how clones were identified to a particular layer. (C) High-power magnification of the cells from (A) showing how the individually stained AP^+ cells were cut out of the tissue. (D) Schematic of the technique used to identify clonally related cells in the gut.

FIG. 2. Schematic of the chicken gut showing questions addressed in this study. (A) Schematic of the adult chicken gut with organs labeled. The different colors correspond to gene-specific expression patterns that have been noticed within the gut from E4 to at least E6. (B) A cross-sectional view through a gizzard showing gene-specific expression within the radial axis of the gut. (C) Schematic depicting the two possible outcomes for the following question: Can clonally related cells cross organ boundaries during development? (D) Schematic depicting the two possible outcomes for the following question: Can clonally related cells cross radial boundaries in the gut during development? Abbreviations: SI, small intestine; LI, large intestine.



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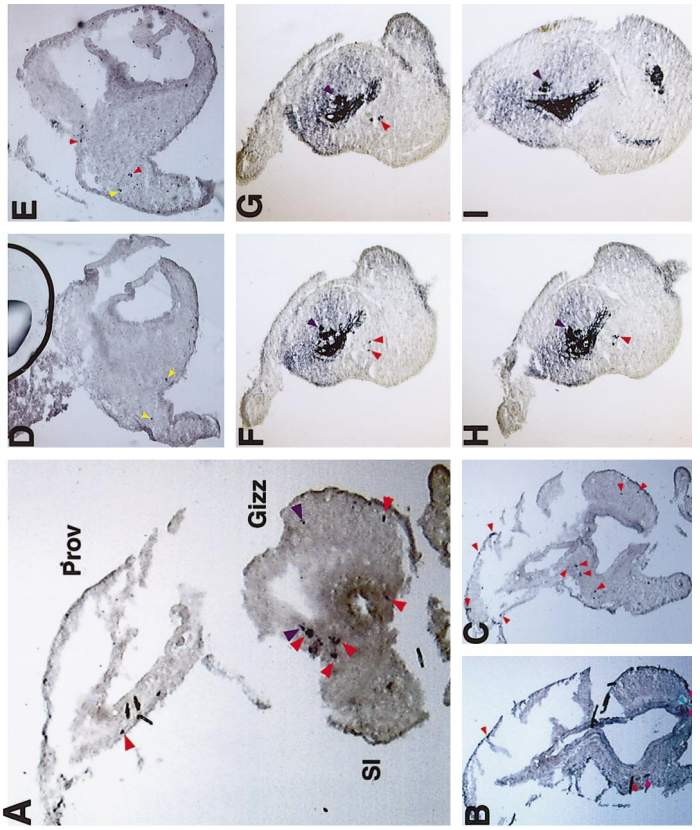

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FIG. 3. Guts injected at stages 8–11 showing the types of clones and staining obtained in selected guts. (A–C) Guts from embryos injected at stage 8. (D–F) Guts from embryos injected at stage 9. (G–I) Guts from embryos injected at stage 11. (J) Gut from an embryo injected at stage 11. Number in the lower right corner is the stage of the embryo when injected with retrovirus. Red arrowhead points to gizzard/small intestinal border, sky blue arrowhead points to clones which have cells located in both deep and superficial layers of the mesoderm, and dark blue arrowhead points to clones containing small numbers of cells.

FIG. 4. Guts injected at stage 8 (A–E) or stage 11 (F–I) showing the lineage relationships of cells in selected sections. Cells indicated with the same color of arrowhead in the same embryo are sibling cells derived from a single progenitor. Abbreviations: Prov, proventriculus; Gizz, gizzard; SI, small intestine.

(1) Can clonally related cells cross organ boundaries, and, if so, is there a temporal restriction in the ability of these cells to cross organ boundaries? (2) Can clonally related cells cross the radial layers of the gut within an organ?

MATERIALS AND METHODS

Embryos and Retroviral Injections

Eggs were obtained from SPAFAS (Connecticut) and incubated to the desired stages (stages 8–20 for this study) (Hamburger and Hamilton, 1951). Eggs were windowed, 5 ml of albumin was removed, and the embryos were injected with the CHAPOL replication-incompetent retrovirus at the desired stages. Retroviral injections targeted the foregut primordia, in particularly the stomach/small intestinal border, as previously described (Roberts *et al.*, 1998). Following injection, embryos were incubated to E6, harvested, and placed into 4% paraformaldehyde for 24 h. The CHAPOL retrovirus was produced, concentrated, and titered, as described (Golden *et al.*, 1995).

Lineage Analysis and Sequencing

Fixed embryos were washed with phosphate-buffered saline and stained for AP activity (Golden *et al.*, 1995). Guts that exhibited AP-positive cells, at the stomach/small intestine border or within the gizzard, were photographed. These guts were then embedded in OCT and sectioned at 20 μm using a Leica cryostat. The sections were evaluated for regions of AP^+ staining and photographed using a digital camera. Cell picking, PCR conditions and amplification, and sequencing of DNA inserts followed the methods of Lin and Cepko (1999).

RESULTS

To address which clonally related cells populate regions that cross organ boundaries and/or span the radial layers of the gut, we used a retrovirally based lineage analysis. If compartment boundaries do exist within the gut, they could be established at any time subsequent to the formation of the primitive gut tube. We, therefore, targeted our injections to a series of time points.

Stage 8–11 Injections

Gut morphogenesis begins at stage 8 with the formation of the AIP (Hamburger and Hamilton, 1951). Morphological distinctions between the future organs of the gut do not become apparent until stage 23 and microscopic and physiologic differences are seen only much later, by E11 (Romanoff, 1960). Hence, injections between those stages infect cells of the early primitive gut tube. Embryos injected at stages 8–11 and harvested at E6 (n = 25) showed two types of clones, groups of spatially contiguous AP^+ cells, apparent in whole mount of histochemically stained foreguts. The first type of putative clone contained numerous cells. Some medium- to large-sized putative clones contained cells that spanned the proventriculus, gizzard, and small intestinal borders (Figs. 3A, 3F, 3I, and 3J), while others appeared restricted to the gizzard only (Figs. 3B, 3C, 3F, and 3H). One interesting feature of the putative clones in these embryos is that the cells appear to be in both deep and superficial layers of the mesoderm within the organ, suggesting that these cells populate different radial layers of the mesoderm (Figs. 3B and 3C). The second type of putative clone was quite small and typically contained just a few cells that were very near to one another (Figs. 3D–3H). These putative clones could be seen in small intestine, gizzard, and proventriculus (Fig. 3). The cells that comprise the small clones also appear to be located in the same radial layer of the mesoderm (Fig. 3).

To verify that the identified clusters of AP^+ cells were clonal in origin, and to determine the cell layer in which each cell lies, the stained guts were sectioned and processed for lineage analysis. Lineage analysis was performed upon six embryos, two injected at stage 8, one injected at stage 9, one injected at stage 10, and two injected at stage 11. A total of 906 AP^+ cells were picked from these embryos, and 362 (40%) of these cells were amplified via PCR and sequenced. Based upon this analysis, many of the large putative clones observed in whole mount were found to indeed be clonal in origin (marked by identically colored arrowheads in Fig. 4). We find that the large clones found in embryos injected from stages 8–11 can span multiple organs, including the esophagus, proventriculus, gizzard, and duodenum (Figs. 4A–4E and data not shown). It is interesting to note that although these clones can span multiple organs, we did not see any clones located in the small intestine posterior to the duodenum following our foregut-targeted injections.

In a stage 8-injected embryo, the cells within small clones are quite distant from one another (Figs. 4A, 4B, 4D, and 4E), in contrast to that seen with a stage 11-injected embryo, in which the cells within small clones are adjacent to one another (Figs. 4F–4I). In most cases, the sister cells of small clones are not located in two different organs; however, one stage 8-injected small clone extends to both the gizzard and the small intestine (Figs. 4D and 4E, yellow arrowheads).

In addition to the clones crossing organ boundaries, clonally related cells were found to span the radial boundaries of the gut. In both the large and the small clones, we find that the cells can be located adjacent to the endoderm and at the outermost layers of the mesoderm (Fig. 4). Therefore, at the stages of injection from 8 to 11, there is little restriction of fate of clonally related cells to organ or radial layer.

Stage 12–16 Injections

Between stages 12 and 16 the foregut remains a simple gut tube. However, embryos injected at stages 12–16 (n = 55) yielded a different pattern of clone dispersion. While in the stage 8- to 11-injected embryos, we see putative clones that span multiple organs, we do not see such clones in the stage 12- to 16-injected embryos (Fig. 5). We find that the

putative clones are localized specifically to the gizzard (Figs. 5A–5F and 5H–5O) or specifically to the small intestine (Figs. 5G and 5P). This was true for both small and large clones. The clones were directly adjacent to an organ boundary. For example, in a number of embryos, AP^+ cells were located within the gizzard and pyloric sphincter adjacent to the small intestine, but no AP^+ cells were found within the small intestine (Figs. 5B, 5F, 5H, 5J, and 5K). This suggests that a restriction of cell migration across this organ boundary occurs between stage 11 and stage 12. Since no putative clones span organ boundaries, it was not necessary to verify the clonal assignments by sectioning, PCR, and sequencing.

We do, however, find the AP^+ cells of a single clone located both deep within the gizzard and superficially upon the gizzard surface (Figs. 5H, 5L, 5M, 5N, and 5O). This suggests that the restriction of daughter cells to particular radial layers has not yet occurred. The fact that no clonal restrictions are established between radial layers was verified at an even later stage of injections (see stage 17–20 injections below).

Stage 17–20 Injections

Between stages 17 and 20 differences in the thickness of various regions of the gut tube become apparent, and by stage 20, budding of gut-derived structures has commenced. For example, the cecal buds form at the small intestine/large intestine border between stages 19 and 20 (Romanoff, 1960). However, we see a similar pattern of clones in embryos injected at stages 17–20 ($n = 16$) in whole mount as we observed in embryos injected at stages 12–16 (data not shown). Lineage analysis was performed upon six embryos (three stage 17 injected, one stage 18 injected, and two stage 20 injected). A total of 780 AP^+ cells were picked from the tissue sections and 312 (36%) amplified via PCR. These amplified PCR products were then sequenced and compared to one another. We find that there are two major clone types. There are clones composed of few cells, <5 cells (a majority of the identified clones), and clones composed of numerous cells, >5 cells (Figs. 6 and 7). The cells in small clones tend to be very close to each other and these cells are restricted to a single radial layer and a single organ (Fig. 6). Such clones can be observed in all radial layers. For example, a small clone in a stage 20-injected embryo contained a few cells, all adjacent to the endoderm (Figs. 6E and 6G, green arrows and purple arrow), while a clone in a stage 17-injected embryo contained only endodermal cells (Fig. 7G, green arrows). In another stage 20-injected gizzard, we also find several small cell clones that are restricted to specific radial layers of the gut (Figs. 6H–6M).

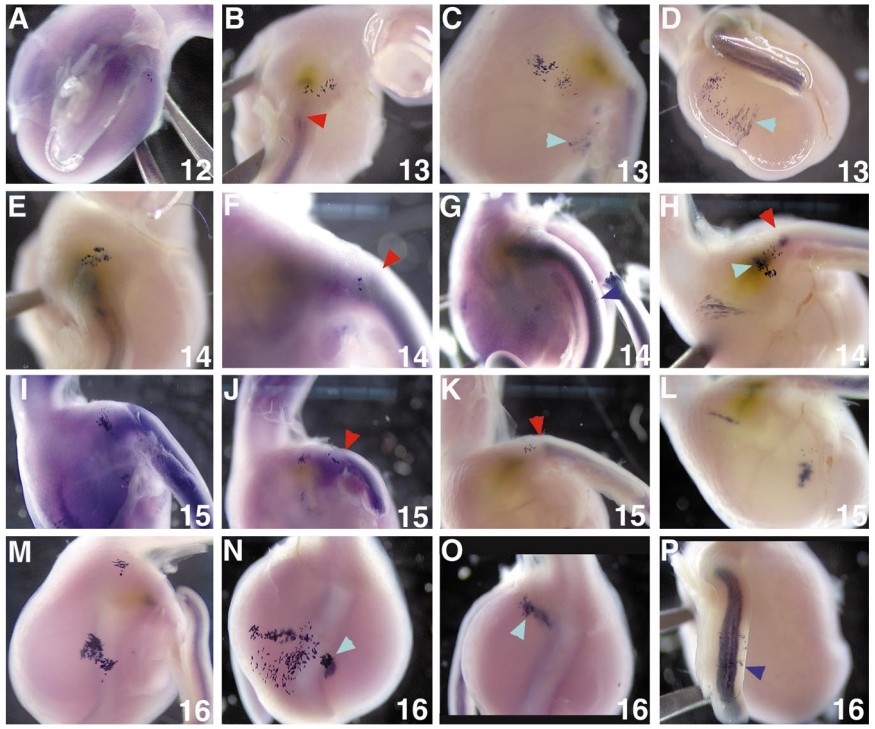
Large clones are also found throughout the injected embryos and can be either restricted or widespread along the radial axis. For example in a stage 17-injected embryo, we find that a single clone is found throughout the submucosa and the muscularis layers of the gizzard (Figs. 7A–7H, dark purple arrows). This suggests that if restriction to a radial layer occurs, it occurs late in development of the gut. We also saw this same pattern in stage 20- and 18-injected embryos (Figs. 6A–6J and data not shown). In other examples of large clones, we find that a stage 18-injected gizzard exhibits two large clones in which most of the identified cells are in a restricted area, while a few scattered members of these clones are found in other regions of the gizzard (Figs. 7I–7Q). The cells of these two large clones in this gizzard span the muscularis and submucosal layers of the gut. Therefore, there is no radial restriction of cells derived from a single progenitor by stage 20. However, as in the stage 12–16 injections, all clones analyzed from injections between stages 17 and 20 were restricted to a single gut organ.

DISCUSSION

The vertebrate gut is a complex structure that is patterned quite early in the development of the embryo (Montgomery *et al.*, 1999). Some of the molecules that play a role in patterning the gut have been identified (Montgomery *et al.*, 1999; Grapin-Botton and Melton, 2000). Many of these patterning molecules are expressed in domains restricted to particular organ primordia (Roberts *et al.*, 1995, 1998; Yokouchi *et al.*, 1995; Smith and Tabin, 1999; Narita *et al.*, 2000). This study has attempted to address whether this restriction of gene expression patterns reflects a restriction of cellular movements of daughter cells between organs. In order to address this question, we have utilized the CHAPOL retroviral vector to study the eventual position of clonally related cells within the gut (Golden *et al.*, 1995).

Temporal Restriction of Distribution of Clonally Related Cells

The results presented here suggest that there is a point at which clonally related cells are restricted to an organ. Before this restriction point, clonally related cells can populate the gizzard, proventriculus, and/or small intestine. Once the restriction point occurs, clones can populate only a single organ. We first observe such a restriction in embryos injected at stage 12. The process of finding and binding of the retroviral particle to the host cell receptor takes place quite rapidly, within 30 min of injection; and internalization of the virion, reverse transcription, and preparation for integration all occur within 2 h of infection. However, the virus cannot integrate until the proper phase of the cell cycle is reached, and it then integrates and, thereby, marks only one of the two daughter cells. This is because integration can occur only after completion of DNA synthesis because of a requirement for nuclear envelope breakdown (Roe *et al.*, 1993). The length of the embryonic chick cell cycle has been measured at 10 h (Fujita *et al.*, 1962), and while this time may vary by stage and tissue, it can be used to estimate an approximate change in developmental stage between infection and irre-



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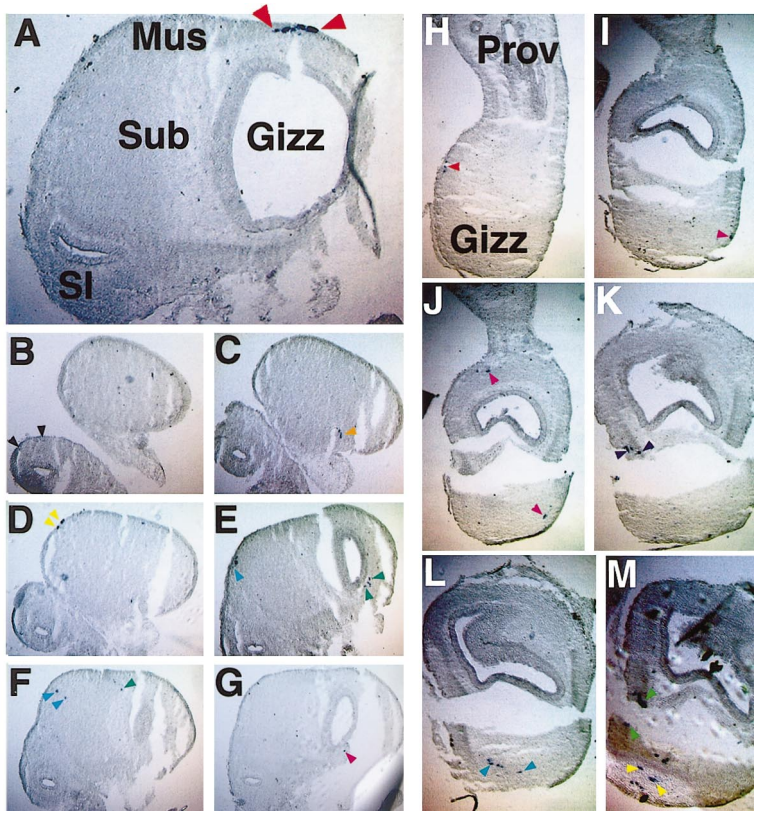

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FIG. 5. Guts injected at stages 12–16 showing the staining and types of putative clones obtained in selected guts. (A) Gut from an embryo injected at stage 12. (B–D) Guts from embryos injected at stage 13. (E–H) Guts from embryos injected at stage 14. (I–L) Guts from embryos injected at stage 15. (M–P) Guts from embryos injected at stage 16. The red arrowhead points to gizzard/small intestinal border, while the light blue arrowhead points to clones which have cells located in both deep and superficial layers of the mesoderm, and the dark blue arrowhead points to a small intestinal-specific clone.

FIG. 6. Guts from two different embryos (A–G and H–M) injected at stage 20 showing the lineage relationships of cells in selected sections. Cells indicated with the same color of arrowhead in the same embryo are sibling cells derived from a single progenitor. Abbreviations: Prov, proventriculus; Gizz, gizzard; Sub, submucosa; Mus, muscularis; and SI, small intestine.

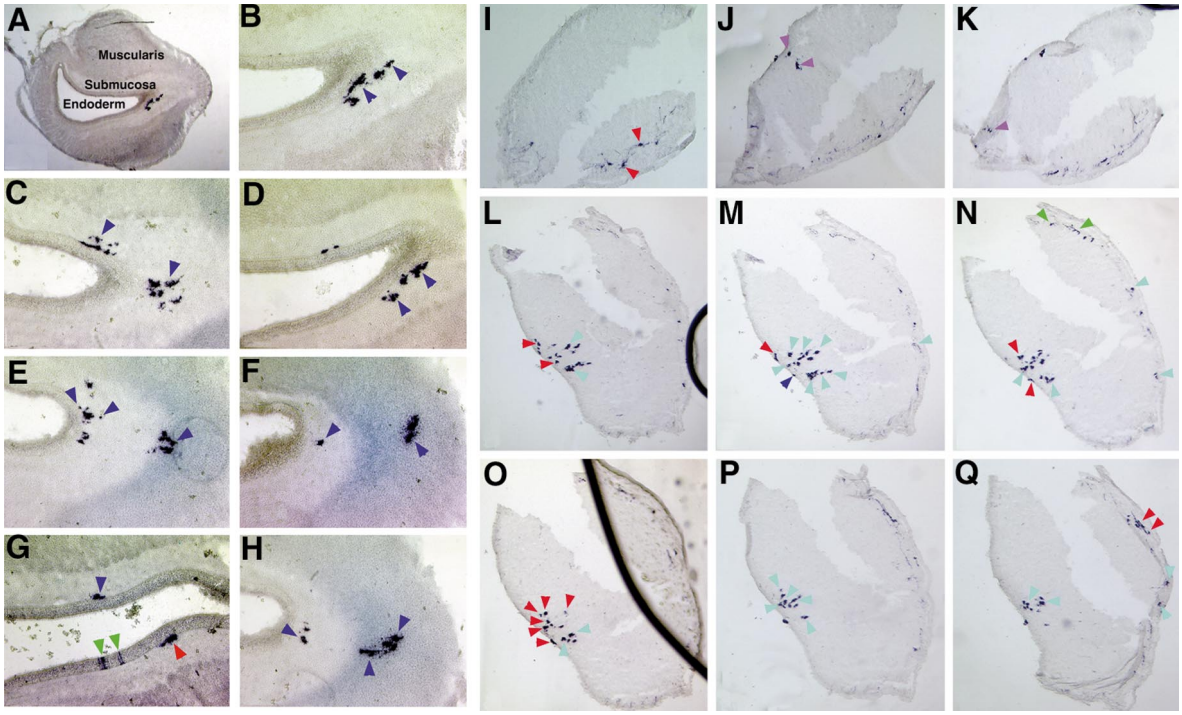

FIG. 7. Guts injected at stage 17 (A–H) and stage 18 (I–Q) showing the lineage relationships of cells in selected sections. Cells indicated with the same color of arrowhead in the same embryo are sibling cells derived from a single progenitor.

vocably marking a single cell. On this basis, 12 h following infection at stage 12, an embryo will have reached stage 15–16 (Hamburger and Hamilton, 1951), which our experiments, therefore, suggest is the approximate time when cells become restricted from transversing the boundaries between gut organ primordia. No such restrictions to cell migration occur along the radial axis within the stages examined in our study.

Progenitor Cells within the Gut

We found two distinct types of clones in the guts. The first type of clone contained very few cells and the cells were very near one another, while the second type of clone contained a much larger number of cells. This distinction was seen at all stages examined. Since there was not a continuum of clone sizes, but rather this clear grouping into two classes, the most parsimonious explanation is that the large clones represent infections of progenitor gut stem cells. The size of these clones provides an estimate of the number of cell divisions which occur following commitment to differentiation. These small clones were all fewer than eight cells in size, suggesting that no more than three cell divisions occur once cells leave the progenitor state. The large type of clone contained large numbers of cells that were located some distance from each other. These clones could cross organ boundaries as well as radial boundaries within the gut. This type of clone was found throughout all of the stages injected, although a restriction to organ boundaries occurred after stage 11. In contrast, the small clones never cross organ boundaries nor radial layers. This suggests that it is the progenitor cells which migrate along both dimensions and that once daughter cells commit to differentiation, they are very limited in their subsequent migration.

Compartment Boundaries within the Gut

Compartment boundaries are important features in development of invertebrates and vertebrates (Dahmann and Basler, 1999). A compartment boundary is a region of an embryo in which cells are restricted to one side of a border. While these cells can freely intermix with cells in their compartment, there is a restriction of cells from mixing with cells of the adjacent compartment (Dahmann and Basler, 1999). In invertebrates compartment boundaries are established by differential gene expression. Many examples of compartments can be cited in the *Drosophila* embryo, including: the eye (Morato and Lawrence, 1978) and the wing (Garcia-Bellido *et al.*, 1973; Morato and Lawrence, 1975). In vertebrates, compartment boundaries have been identified in the hindbrain (Fraser *et al.*, 1990; Birgbauer and Fraser, 1994) and in the chicken limb ectoderm (Altabef *et al.*, 1997). Similar molecules have been implicated in com-

partment boundary formation in vertebrates and invertebrates, such as the Hh family of secreted proteins and the Engrailed transcription factors (Dahmann and Basler, 1999).

Recently, the Eph family of molecules has been implicated in restricting cells into compartments in the vertebrate hindbrain (Mellitzer *et al.*, 1999). Thus, the differential expression of an EPH ligand and an EPH receptor in two adjacent compartments within the hindbrain results in the compartmentalization of the cells and the prevention of any cell mixing between the two compartments (Mellitzer *et al.*, 1999). This result with the Eph family of proteins is intriguing given the results in the current study. Here we find that compartment boundaries are present in the gut and prevent the mixing of clonally related cells after early stages of development. We have also found that the Eph molecules are differentially expressed within the gut. This is based upon antibody staining of both of the A and B receptor and ligand classes (Gale *et al.*, 1996). We find that the boundaries of expression of the Eph A and B receptors and ephrin A and B ligands correlate with the border of the small intestine and the stomach starting at stage 19–20 (data not shown). Thus, the restriction of clonally derived cells to distinct organ primordia could be due to the upregulation and appearance of the Eph proteins.

Setting Up Compartment Boundaries within the Gut

We have focused upon the compartment boundary between the gizzard and the small intestine. Here we show that clonally related cells marked by infections after stage 11 cannot cross the border between the two organs. Therefore, the regionalization of the gut must happen very early in development, by approximately stage 15–16. Studies have shown that the Hox genes are responsible for organ-specific patterning events and are regionalized early in gut development to organ-specific regions (Roberts *et al.*, 1995; Yokouchi *et al.*, 1995; Warot *et al.*, 1997; Zakany and Duboule, 1999). The Hox code within the gut is set up by stage 13–14 in the gut (Roberts *et al.*, 1995), which correlates well with our lineage data. Therefore, Hox genes could play a role in forming the compartment boundaries of the gut. It is possible that the Hox genes impose a pattern in which the Eph boundaries of expression will occur. Thus, the Hox proteins could set up the organ regionalization and the EPH proteins could enforce the compartment borders between them. Additional studies of the Hox genes and the Eph genes should yield more insight into the patterning of the gut and the role of these genes in setting up and regulating compartment boundaries.

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