Region-Specific Expression of Chicken Sox2 in the Developing Gut and Lung Epithelium: Regulation by Epithelial-Mesenchymal Interactions

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ABSTRACT In situ analysis of the chicken cSox2 gene, a member of the transcription factor family containing an Sry-like high-mobility group (HMG) box, demonstrated localized expression in the embryonic endoderm. Transcripts of cSox2 appeared before commencement of morphogenesis and cytodifferentiation in the rostral gut epithelium from the pharynx to the stomach. The caudal limit of cSox2 expression coincided with that of the region competent for proventricular differentiation and to the rostral limit of the domain of CdxA, a homologue of Drosophila caudal. During morphogenesis, the level of transcripts of cSox2 decreased in epithelia invaginating into surrounding mesenchyme to form glandular or tubular structures, such as the primordia of the thyroid and lung, glandular epithelium of the proventriculus, and secondary bronchus of the lung. Tissue recombination experiments demonstrated that cSox2 expression is regulated by the underlying mesenchyme as well as morphogenesis and cytodifferentiation. The results suggest that cSox2 plays pivotal roles in generating morphologically and physiologically distinct types of epithelial cells in the gut. Dev. Dyn. 1998;213:464–475. © 1998 Wiley-Liss, Inc.

Key words: chicken embryo; Sox gene; HMG box; endoderm; CdxA; regionalization; organogenesis; tissue interactions

INTRODUCTION The high-mobility group (HMG) domain is a DNA binding motif that was originally discovered in abundant nonhistone components of chromatin. The proteins containing this motif are considered as architectural components in the assembly of nucleoprotein complexes, and they regulate transcription by interacting with the minor groove of the DNA helix and modulating DNA structure by bending the DNA helix (reviewed in Grosschedl et al., 1994). Proteins containing a single HMG domain include the lymphoid enhancer-binding factors LEF-1 and TCF-1, the testis-determining factor SRY, and closely related SOX proteins. These factors recognize specific nucleotide sequences (Denny et al., 1992b; Harley et al., 1992, 1994; Travis et al., 1991; van de Wetering et al., 1991; Waterman et al., 1991) and work in specific cell types. At least 20 Sox genes have so far been identified in mammals and more in other vertebrates and invertebrates, such as Xenopus (Denny et al., 1992a; Hudson et al., 1992a, 1992b; C. elegans (Denny et al., 1992a, 1992b), and Drosophila (Nambu and Nambu, 1996; Russell et al., 1996). They have been implicated in the regulation of a variety of developmental processes. Sox9 is a causal gene of campomelic dysplasia and is involved in control of both testis development and bone formation (Foster et al., 1994). Sox-5 is expressed in postmeiotic germ cells in adult testis (Denny et al., 1992b). Sox-4 encodes a transcriptional activator expressed in lymphocytes (van de Wetering et al., 1993). A Sox gene in Drosophila is required for embryonic segmentation and nervous system development and its regulatory role is to modulate expression of pair-rule genes (Nambu and Nambu, 1996; Russell et al., 1996). In the chicken, cSox2, cSox3, cSox11 are expressed in the central nervous system (CNS) in patterns that are closely associated with maturation of neurons: cSox2 and cSox3 are predominantly expressed in the immature neural epithelium, while cSox11 expression is transiently upregulated in maturing neurons (Uwanogho et al., 1995; Stréat et al., 1997). In the earliest stages of neural development, cSox3 is expressed in the epiblast that is competent to neural induction, while cSox2 transcripts then appear in the CNS just after neural induction (Rex et al., 1997b). In Xenopus, Sox-2 has been shown experimentally to be an early responsive gene to Chordin, a neural inducing factor, and to modulate responsiveness to other extracellular signals (Mizuseki et al., 1998). Kamachi et al. (1995, 1998) have shown that the Sox2 product binds to Δ1-crystallin enhancer and regulates its activity in the lens (Kamachi et al., 1995, 1998), suggesting late roles of Sox genes during organogenesis.
A central issue of developmental biology is how regional differentiation and morphogenesis of embryonic tissues are brought about. It is well established that interactions between different tissues play important roles in these processes. The gut is an attractive system to study such interactions since its epithelium exhibits rostrocaudally distinctive features which have been well characterized. The gut is roughly divided rostrocaudally into esophagus, stomach, and intestine. In birds, the stomach consists of two portions, the proventriculus (the glandular stomach) and gizzard (the muscular stomach), distinguishable from each other both morphologically and physiologically. The proventriculus is a fusiform enlargement situated at the lower end of the esophagus. Its epithelium forms numerous compound glands which produce digestive juice containing pepsinogen, the zymogen of pepsin. Gizzard epithelium does not form any complex glands and secretes no digestive enzymes and its function in digestion is a mechanical one. The lung epithelium is also derived from the endoderm and continuous with that of the digestive tract.

Regional differentiation and morphogenesis of the gut epithelium requires tissue interactions involving instructive effects of mesenchyme and competence of the epithelium receiving them (Yasugi, 1993, 1994). Proventricular mesenchyme can induce esophageal, proventricular, and gizzard epithelia to form proventriculus-type glands which secrete embryonic chicken pepsinogen (ECPg). These epithelia, however, never form such glands when cultured with gizzard mesenchyme (Takiguchi et al., 1986; Urase et al., 1996). Although small intestinal and allantoic epithelia form glandular structures when cultured with proventricular mesenchyme, they do not produce ECPg antigen (Yasugi et al., 1985). Intestinal epithelium loses the competence to express ECPg before closure of the digestive tube (Yasugi et al., 1991). As for the establishment of regionalization of the gut, it is suggested that the differential expression of Sonic hedgehog, endodermally derived signalling molecule, controls the fate of the different region of the gut mesenchyme (Roberts et al., 1995; Apelqvist et al., 1997).

Regional specification of the embryonic body appears to involve transcription factors which regulate sets of genes required for specific developmental pathways. Progress in understanding function and regulation of such genes in the endoderm will provide insights into how the endoderm generates different phenotypes from different regions of the gut. Until recently, few molecular markers have been identified that may be involved in early events of gut development. The Abd-B subfamily of the HoxA cluster genes are expressed in the caudal gut with nested rostral limits in the small intestine and more caudal regions such as ceca and large intestine (Yokouchi et al., 1995). Hoxd-13 is also expressed in the caudal gut mesoderm and regulates the caudal phenotype of the gut endoderm (Roberts et al., 1998). CdxA, a homologue of the caudal gene in Drosophila, is expressed solely in intestinal epithelium (Frumkin et al., 1991, 1994). More recently, we have found that the restricted expression of CdxA in intestinal epithelium can be traced back to early phases of digestive tube formation (Ishii et al., 1997). Our data from tissue recombination experiments strongly suggest that the rostral limit of CdxA expression after the digestive tube formation is determined by mesenchymal influences. As well as the gut, epithelial-mesenchymal interactions are important for branching morphogenesis of the lung (Dameron, 1961; Aliesco and Cassini, 1962; Spooner and Wessells, 1970).

In the current study, we examined Csox2 expression in the developing gut and lung. Csox2 is expressed in the rostral gut epithelium in a fashion reciprocal to CdxA expression. We also show that expression of Csox2 is switched off in epithelia which are actively changing their morphology. Tissue recombination experiments demonstrate that Csox2 expression is regulated by mesenchymal influences. Our results demonstrate that Csox2 expression is involved in early regionalization closely associated with response to mesenchymal signals and morphogenesis and/or cytodifferentiation during organogenesis.

**RESULTS**

Csox2 Expression During Formation of the Digestive Tube

Csox2 expression was not detected in the endoderm before Hamburger and Hamilton (1951; HH) stage 8. Thus, in the early embryo, cells of the epiblast were positive for Csox2 expression but expression was lost as cells entered the primitive streak and migrated ventrally to form the mesoderm and endoderm (Fig. 1A). From HH stage 7 the endoderm begins to form the anterior intestinal portal (AIP). By HH stage 8 the AIP is well developed and opens out at approximately the level of the developing heart a little way rostral to the first somite. Csox2 transcripts were first detected at the four- to five-somite stage (late HH stage 8) in a very restricted domain of the endoderm around the AIP.

Fig. 1. (Overleaf.) In situ hybridization for Csox2 expression. Hamburger and Hamilton (1951; HH) stage of embryos as indicated in bottom right corner. No Csox2 expression is seen in the hypoblast at HH stage 6 as shown in a transverse section of wholmount-stained embryo, in which Csox2 expression is restricted to the epiblast (A). By HH stage 8, Csox2 expression is detected in a restricted domain of the endoderm around the anterior intestinal portal (AIP) on either side (B–D). C and D are transverse sections of wholmount-stained embryos from approximate levels of AIP (C) and the first somite (D). As the foregut continued to extend from the AIP (during HH stages 9 and 10), Csox2 is expressed throughout the mediolateral region that forms the digestive tube (E, F–I) are transverse sections from more caudal regions of a HH stage 15 embryo wholemount-stained for Csox2 expression. Arrowheads indicate limits of Csox2 expression. Note that the domain of Csox2 expression defines exactly the region that forms digestive tube, whereas the endoderm which comes to lie outside of the digestive tube is Csox2-negative even before the tube actually forms. Csox2 expression appears only a short distance rostral to the AIP; more caudal endoderm is negative (I). Scale bars ~ 100 μm.
Figure 1. (Legend on previous page.)
Fig. 2. Embryos of 3 days of development (HH stage 16–17) stained for cSox2 expression by in situ hybridization on tissue sections. A: cSox2 expression on a horizontal section of HH stage 17 embryo through the rostral part of the gut. cSox2 expression is seen in the gut epithelium from the pharynx to the stomach. Outside of the gut, expression was seen throughout the neural tube at a higher intensity. B: Higher magnification of boxed region of A around the gut epithelium. C: Higher magnification of boxed region of A around the thyroid (th) which is negative for cSox2 expression. D: Sagittal section of an embryo of HH stage 16 through the initial bud of the lung (lu) which shows a low level of cSox2 expression. st, stomach. Scale bars = 1 mm for A; 100 µm for B–D.

Fig. 3. Rostrocaudal limits of cSox2 (A,C) and CdxA (B,D) expression in 4-day (A,B) and 10-day (C,D) gut epithelium. In 4-day gut, cSox2 is highly expressed in the gizzard (gz) and its expression gradually decreases toward duodenum (du) where CdxA is expressed. Both genes are expressed at low levels in a small region of overlap. Arrows indicate the position where the bile duct opens out. By day 10 of development, gizzard-duodenal boundary becomes evident as a fold of the rostral limit of previllous ridge (arrowheads). cSox2 is expressed at a high level only in the region rostral to this fold whereas CdxA is expressed only in the caudal region where cSox2 expression is very low. Scale bars = 100 µm.
extending a little way caudal in the endoderm on either side (Fig. 1B–D).

As the foregut continued to extend from the AIP (during HH stages 9 and 10), cSox2 was expressed throughout the mediolateral region that forms the digestive tube (Fig. 1E; see also Fig. 10), which became even more evident by HH stage 15. In these older embryos, expression of cSox2 continued to appear just caudal to the AIP where expression was precisely restricted to the medial region of endoderm that folded and fused to form the digestive tube (Fig. 1F–I). Thus, cSox2 expression appeared to demarcate the prospective digestive tube endoderm a short time before completion of folding and fusion of the splanchnopleures.

Expression of cSox2 Defines Rostral Gut Epithelium

We analyzed the spatial and temporal distribution of cSox2 transcripts in the developing gut after its closure by in situ hybridization on tissue sections.

Transcripts of cSox2 were detected in the epithelium of the rostral gut on day 3 (HH stage 16–17) of development (Fig. 2A,B). On day 4, transcripts of cSox2 were abundant in the gut epithelium rostral to the stomach and they gradually decreased caudally toward the caudal part of the duodenum where the bile duct opens out (Fig. 3A). Comparing the expression of cSox2 with that of CdxA on adjacent sections revealed that cSox2 was expressed complementarily to CdxA with slight overlap in the duodenum. In the region of overlap, the expression of both was weak (Fig. 3A,B). By day 10 of development, as the boundary between gizzard and intestine became evident as a fold, a high level of cSox2 expression was confined in the epithelium rostral to this fold, which exactly coincided with the rostral limit of CdxA expression (Fig. 3C,D). After day 12 of development, the expression of cSox2 declined especially caudally. By day 15, cSox2 expression was absent from gizzard epithelium, weak in proventricular epithelium, but maintained at high levels in esophageal epithelium (data not shown).

cSox2 Expression During Morphogenesis

The level of cSox2 expression changed dramatically in a variety of epithelia undergoing extensive morphogenesis. On day 3 of development, primordia of some organs could be recognized as invaginations of epithelium and they exhibited reduced expression of cSox2. The thyroid primordium growing away from the median floor of the pharynx was negative for cSox2 transcripts (Fig. 2C). The initial primordium of the lung expressed cSox2 at a significantly lower level than the neighboring endoderm of the pharynx and esophagus (Fig. 2D).

The epithelium of the proventriculus begins to form glands on day 6 of development. At this stage, cSox2 expression decreased in some clusters of proventricular epithelial cells just as they began to invaginate into the surrounding mesenchyme (Fig. 4A). Subsequently, as the glandular epithelium elongated into the surrounding mesenchyme, the level of cSox2 expression remained lower than in the luminal epithelium (Fig. 4B). Expression of ECPg was detected in the glandular epithelium where cSox2 expression was low (Fig. 4C,D). By day 15, cSox2 expression declined in the luminal epithelium to a level almost equal to that in the glandular epithelium (data not shown).

The morphogenesis of the lung is accomplished by the outgrowth and repetitive branching of the primary bronchi which run through the bifurcated bud, a process known as branching morphogenesis. The change in cSox2 expression was also observed during this morphogenetic event. In 4-day embryos, before branching morphogenesis took place, cSox2 was expressed intensely and uniformly in the primary bronchi except in their distal ends where no expression was seen (Fig. 5A). By day 6 of development, cSox2 expression ceased in the distal tips of the secondary bronchi sprouting out from the primary bronchus (Fig. 5B). The epithelium of primary bronchus remained positive for cSox2 expression until at least day 9 of development.

cSox2 Expression in Tissue Recombinants

To analyze whether cSox2 expression is regulated by signals from the mesenchyme, tissue recombination experiments were carried out (Fig. 6). Epithelia were isolated from 4-day embryos before commencement of morphogenesis and cytodifferentiation. Mesenchymes were isolated from 6-day embryos. Epithelia and mesenchyme were recombined in various combinations and cultured in vitro.

Fig. 6. The schematic diagram of tissue recombination experiments. Epithelia before commencement of morphogenesis were isolated from the gut or lung dissected from 4-day embryo. Mesenchymes were isolated from 6-day embryo. Epithelium and mesenchyme were recombined in various combinations and cultured in vitro.
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When 4-day stomach epithelium was cultured with 6-day proventricular mesenchyme, the epithelium formed numerous glandular structures, such as normal proventriculus, in which the level of cSox2 was low and ECPg was actively expressed (Fig. 7A,B). When cultured with gizzard mesenchyme, stomach epithelium expressed cSox2 uniformly at a high level and neither glandular structures nor ECPg expression were seen (Fig. 7C,D). Thus, the pattern of cSox2 expression, as well as epithelial morphology and cytodifferentiation, reflected the source of mesenchyme.

Further experiments revealed that mesenchyme of the gizzard and the small intestine, situated adjacent to the caudal limit of cSox2 expression, exert effects that direct epithelium to express cSox2 in a mesenchyme-dependent manner. When stomach epithelium was cultured with small-intestinal mesenchyme, some clusters of the epithelial cells exhibited reduced expression of cSox2 (Fig. 8A). Some of these clusters expressed CdxA (Fig. 8B) and sucrase antigen (not shown), a marker for intestinal differentiation (Matsushita, 1985). Other clusters where cSox2 expression was low expressed ECPg, consistent with previous studies in which small-intestinal mesenchyme allows ECPg expression in small areas (Urase et al., 1996; Ishii et al., 1997). When small-intestinal epithelium was cultured with gizzard mesenchyme, cSox2 expression was induced, occasionally in large area of the epithelium (Fig. 8C). CdxA expression was suppressed in the epithelium where cSox2 was induced (Fig. 8D). In homotypic recombinants composed of small-intestinal epithelium and small-intestinal mesenchyme, cSox2 was never expressed and CdxA expression was maintained as seen in normal development of the intestine (data not shown).

The effect of proventricular mesenchyme on small-intestinal epithelium differs from that of gizzard mesenchyme. Small-intestinal epithelium never expressed cSox2 and extensively expressed CdxA when cultured with proventricular mesenchyme (Fig. 8E,F). Thus, the ability of mesenchyme to induce cSox2 expression and to suppress CdxA expression is present only in the gizzard, the organ situated next to the intestine, but not in the proventriculus. ECPg expression was never detected in recombinants composed of intestinal epithelium and any kind of mesenchyme. The expression of genes in tissue recombinants were summarized in Table 1.

In the lung, secondary bronchi lose cSox2 expression when they sprout out from the primary bronchus. To examine whether lung mesenchyme share a common mesenchymal factor(s) with the proventricular mesenchyme that causes downregulation of cSox2 and changes in epithelial morphology, we compared inductive properties of mesenchymes between proventriculus and lung by homotypic or heterotypic recombinations. When stomach epithelium was cultured with proventricular mesenchyme, the epithelium formed glandular structures that express cSox2 at a low level and express ECPg intensely (Fig. 7A,B). When stomach epithelium was cultured with lung mesenchyme, cSox2 expression was low in wide area of the epithelium (Fig. 9A). ECPg was expressed in epithelium where cSox2 expression was low (Fig. 9B). Recombinants composed of lung epithelium and lung mesenchyme formed vesicular structures, reminiscent of the secondary bronchus, projecting toward circumference of recombinants. In these structures, cSox2 expression was lost completely (Fig. 9C, arrows). When cultured with proventricular mesenchyme, lung epithelium developed into thick and simple epithelium expressing cSox2 uniformly at a high level, and neither glandular nor vesicular structure was observed (Fig. 9D).

### DISCUSSION

Although many classical experimental works have revealed that interactions between epithelium and mesenchyme play crucial roles in morphogenesis and cytodifferentiation of the gut epithelium (reviewed in Yasugi and Mizuno, 1990; Yasugi, 1993, 1994, 1995), the molecular mechanisms that control this process are not known. Sox genes compose a gene family encoding transcriptional factors that regulate expression of specific target genes. We report here spatially and temporally restricted distribution of transcripts of the cSox2 gene in the chicken embryonic gut and lung epithelium. Our results suggest that cSox2 plays pivotal roles in region-specific differentiation and morphogenesis of the endoderm.

### cSox2 Expression and Competence to Respond to Mesenchymal Signals

We revealed that cSox2 expression is expressed in a rostral region of the gut epithelium. Epithelia of the esophageal, proventriculus, and gizzard highly expressed cSox2, while intestinal epithelium exhibited no or very weak signal. Studies on epithelial-mesenchymal interactions...
mal recombinants or transplants have shown that epithelia of the rostral organs, i.e., esophagus, proventriculus, and gizzard, are capable of expressing ECPg when cultured with proventricular mesenchyme, while epithelia of the intestine and allantois never express ECPg and undergo intestinal differentiation (Yasugi, 1984; Yasugi et al., 1985; Hayashi et al., 1988). The caudal limit of cSox2 expression coincided with that of the competence to express ECPg. Furthermore, intestinal epithelium did not express cSox2 when it was cultured with proventricular mesenchyme. Thus, cSox2 expression appears to correlate closely with the competence of the gut epithelium to respond to the influence of proventricular mesenchyme.

Fig. 4. cSox2 expression in the proventriculus in 6-day (A), 9-day (B), and 12-day (C) embryo. D shows the expression of embryonic chicken pepsinogen (ECPg) detected on a section adjacent to C. The level of cSox2 expression in the glandular epithelium (ge) is lower than the luminal epithelium (le) from day 6, just after the initiation of the invagination of glandular epithelium. ECPg is expressed in the epithelia which exhibit weaker cSox2 signals. Scale bar = 100 µm for A–D.

Fig. 5. cSox2 expression in the lung. A: The expression of cSox2 in the 4-day lung stained by whole mount in situ hybridization. B: Transverse section of 6-day lung at the level of invagination of the secondary bronchus. The expression of cSox2 is seen in the primary bronchus and the stalk of the secondary bronchus but not in distal portion of the secondary bronchus. pb, primary bronchus; sb, secondary bronchus. Scale bars = 500 µm for A and 100 µm for B.
One of the most striking features of expression of cSox2 is that its caudal limit almost coincided with the rostral limit of CdxA expression. It seems possible either that cSox2 may negatively regulate CdxA expression or vice versa, since only weak expression of either gene is seen in the narrow region of overlap at early stages (Fig. 3A,B). A possible consequence of these expression domains is the competence of endoderm to respond to mesenchymal signals, as discussed above.

Regional Specification in Early Endoderm

It has been known that endodermal epithelium of 1.5-day embryo demonstrates some regionality which can be visualized by determination of prospective fate map (Matsushita, 1996), by comparison of reactivity to mesenchymal influences (Yasugi et al., 1991) and by a test of autodifferentiation potency (Sumiya, 1976). Recent works including the present one revealed that it is possible to characterize each region in terms of expression of some regulatory genes. In 1.5-day embryo, cSox2 was expressed in the endoderm in the foregut (not shown) and around the AIP (Fig. 1B,C) but not in the caudal endoderm (Fig. 1D). Compared with the fate map of 1.5-day endoderm (Matsushita, 1996), we can postulate that cSox2 is expressed in the anlage of the esophagus, stomach, and anterior part of the duodenum (Fig. 10).

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We have previously reported that CdxA is expressed in the bilateral regions of the caudal endoderm in 1.5-day embryo (Ishii et al., 1997; Fig. 10) which roughly corresponds to presumptive jejunal epithelium. The domain of CdxA expression excludes the medial endoderm of the presumptive dorsal pancreas.
As the region of CdxA expression rapidly extends caudally, the medial part of the presumptive intestinal area, which contains a small number of dorsal pancreatic cells (Matsushita, 1996), also remains negative at least by HH stage 12.

Murine pdx1, a homeobox gene required for pancreatic development, is expressed before the dorsal and ventral pancreatic evaginations appear (Jonsson et al., 1994; Offield et al., 1996). A recent study has shown that several markers of the dorsal pancreas, including the pdx1 gene, are expressed in the chicken pancreatic endoderm from rather early stages (Kim et al., 1997). The absence of transcripts of CdxA in medial endoderm may reflect the commitment of the dorsal pancreatic anlage preceding initial morphogenesis of the pancreas.

Expression of these genes may thus delimit distinct domains before gut closure. The endoderm can be divided into at least three domains which have different molecular landmarks: (1) the rostral region in which cSox2 is expressed, (2) the ventrocaudal region in which CdxA is expressed, and (3) the dorsocaudal region in which neither CdxA nor CdxA is expressed but early pancreatic genes may be expressed. Functional analysis of regulatory genes will provide a clue to examine whether gene expressions truly delineate key biological variables within the early endoderm that are important for generating a variety of cell types.

Spooner and Wessells (1970) demonstrated with mammalian embryo that the initial bud of the lung is formed when early endoderm was cultured alone without mesoderm. We demonstrated that epithelium of the initial bud of the lung expresses cSox2 at a level lower than gut epithelium. In tissue recombination experiments, 4-day stomach epithelium formed proventricular gland under the influence of the mesenchyme of the proventriculus or lung, while lung epithelium did not. Instead, lung epithelium formed cSox2-negative bronchi under the influence of lung mesenchyme. These facts indicate that the lung epithelium and gut epithelium which are derived from common origin are distinguishable in reactivity to mesenchymal influences and gene expression from rather early stages of development.

A Role for cSox2 in Epithelial Morphogenesis

A dramatic change in cSox2 expression was observed when the epithelial morphogenesis occurs. In the proventriculus, cSox2 was downregulated just after commencement of gland formation. No signal for cSox2 expression could be detected in the primordium of the thyroid. In the lung, after cSox2 was once downregulated in its initial bud, it was upregulated again as the lung elongated and formed the bifurcated primary bronchi. During branching morphogenesis, cSox2 expression decreased to an undetectable level at tips of secondary bronchi sprouting out from the primary bronchi. Thus, loss of cSox2 expression appears to closely correlate with the commencement of morphogenesis in a wide variety of organs consisting of endoderm-derived epithelium. It is possible that cSox2 regulates a set of genes which cause cell rearrangements involving breaking and remaking of contact between individual cells. In proventricular glands, several genes such as fra-2 (Matsumoto et al., 1998), sonic hedgehog (Narita et al., 1998), cytokeratin (Sato and Yasugi, 1997), and the chicken spasmolitic polypeptide (Tabata et al., 1998) also ceased to be expressed. It is tempting to speculate that decreased expression of cSox2 is primarily responsible for the regulation of expression of these genes.

Mesenchymal Regulation of cSox2 Expression in the Gut and Lung

Influences derived from the underlying mesenchyme are important for morphogenesis and cytodifferentiation of the epithelium specific to each digestive organ. Proventricular and gizzard epithelia undergo formation of glandular structures and express ECPg when they are cultured under the influence of proventricular mesenchyme but not of gizzard mesenchyme (Takiguchi et al., 1986; Urase et al., 1996). Gizzard mesenchyme can alter the developmental fate of small-intestinal epithelium to that of the gizzard (Matsushita, 1995). Intestinal mesenchyme induces stomach epithel-
lium to differentiate into intestinal epithelium with a striated border, goblet cells, and sucrase production (Ishizuya-Oka and Mizuno, 1984).

The present results of tissue recombination experiments (Table 1) demonstrated that the surrounding mesenchymes of different digestive organs differently regulate cSox2 expression in the epithelium. We have shown that gizzard mesenchyme can induce cSox2 expression and suppress CdxA expression in intestinal epithelium. In contrast, small-intestine mesenchyme exerts influence on stomach epithelium that induces CdxA and suppresses cSox2 expression. A high level of cSox2 expression was seen only in the epithelium where CdxA expression was low, again indicating close correlation between expression of these genes. Mesenchymal actions regulating cSox2 and CdxA may be involved in mechanisms which ensure boundary between gizzard and small intestine.

Proventricular mesenchyme and gizzard mesenchyme exerted different effects on stomach epithelium. Proventricular mesenchyme allowed stomach epithelium to form glands of low expression of cSox2 but gizzard mesenchyme did not. Since lung mesenchyme can also induce differentiation of proventricular glands in the stomach epithelium (Fig. 9A, B; Urase et al., 1996), it may share common or very similar factor(s) with proventricular mesenchyme.

It is an urgent problem to know what molecules are responsible for the induction of proventricular glands. Bone morphogenetic protein (BMP) 2 is a good candidate for the factor which stimulates proventricular differentiation, since its transcripts can be detected in the mesenchymes of the proventriculus and lung.

In the lung morphogenesis, many secreted factors are implicated. Potentially important factors expressed in lung mesenchyme include members of fibroblast growth factor, transforming growth factor-β, BMP, and epidermal growth factor families (Goldin and Opperman, 1980; Peters et al., 1994; Serra et al., 1994; Nogawa and Ito, 1995; Bellusci et al., 1996; Zhou et al., 1996). The roles of BMP and other factors on differentiation and morphogenesis of gut and lung epithelium and their relation to cSox2 and/or CdxA expression should be studied in future.

**EXPERIMENTAL PROCEDURES**

**Animals**

Embryos of White Leghorn chicken (Gallus gallus domesticus) were used and staged according to Hamburger and Hamilton (1951) or days after the beginning of incubation.

**In Situ Hybridization**

A clone with an insert of 1.4 kb cDNA which contains the full length of coding region of cSox2 gene (Uwanogho et al., 1995) was used for preparing digoxigenin-labelled antisense and sense riboprobes. Other probes used were CdxA (618 bp of 3' noncoding region, Ishii et al., 1997) and ECPg (cBg150, Hayashi et al., 1988).

In situ hybridization on frozen sections was carried out according to the method previously described (Ishii et al., 1997) with slight modifications. Tissues were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) overnight at 4°C and embedded in OCT compound. Frozen sections of 12 µm were cut in a cryostat. After rehydration in phosphate-buffered saline containing 0.1% Tween-20, the sections were digested with 1 µg/ml of proteinase K for 7 min at 37°C and postfixed with 4% paraformaldehyde in PBS for 20 min. Hybridization was carried out overnight at 70°C in hybridization solution (50% formamide, 5 × standard saline citrate (SSC; pH 4.5), 1% sodium dodecylsulfate (SDS), 50 µg/ml yeast RNA, 50 µg/ml heparin) containing 1 µg/ml of digoxigenin-labelled RNA probe. The slides were washed twice with 50% formamide, 5 × SSC (pH 4.5), 1% SDS, for 30 min at 65°C and subsequently three times with 50% formamide, 2 × SSC; pH 4.5) for 30 min at 65°C. After washing with Tris-buffered saline containing 0.1% Tween-20 (TBST), the slides were incubated in blocking solution (0.5% blocking reagent [Boehringer Mannheim, Indianapolis, IN] in TBST) for 1 hr, then overlaid with anti-digoxigenin Fab-alkaline phosphatase conjugate diluted to 1/2,000 with blocking solution and placed at 4°C overnight. After three washes with TBST containing 2 mM of levamisole each for 20 min, coloring reaction was carried out with 35 µg/ml nitroblue tetrazolium, 17.5 µg/ml 5-bromo-4-chloro-3-indolyl phosphate in NTMT (100 mM Tris-HCl [pH 9.5], 100 mM Tris-HCl [pH 9.5], 100 mM NaCl, 50 mM MgCl2, 0.1% Tween-20, 2 mM levamisole).

**Wholemount In Situ Hybridization**

The method was as described in Rex et al. (1997a). Embryos stained were refixed in 4% paraformaldehyde and embedded in wax and sections of 10–15 µm were cut.

**Tissue Recombination Experiments**

Epithelium of digestive organs and lung of 4-day embryos were separated from mesenchymes by the treatment of collagenase (Cooper Biochemical, Code CLS, 0.03% in Tyrode’s solution for 30–60 minutes at 37°C) and reassociated with 6-day mesenchymes similarly isolated. Homotypic or heterotypic epithelial-mesenchymal recombinants were then cultured in vitro on the surface of the liquid medium (1:1 solution of 13-day embryo extract and Earle’s 199) for 6 days as previously described (Takiguchi et al., 1988; Urase and Yasugi, 1993). After cultivation, recombinants were subjected to in situ hybridization or immunohistochemistry (for detecting sucrase antigen; Ishii et al., 1997) on frozen sections.

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