

Mesenchymal regulation of epithelial gene expression in developing avian stomach: 5'-flanking region of pepsinogen gene can mediate mesenchymal influence on its expression

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SUMMARY

The expression of a gene encoding an embryonic chick pepsinogen was investigated in developing avian gut. Expression is restricted to the epithelial layer of the embryonic proventriculus (glandular stomach). We can therefore regard this gene as a marker gene for proventricular epithelial differentiation. There is some considerable evidence in favour of epithelial-mesenchymal interactions being important during the development of the gastrointestinal system; for example, pepsinogen expression is induced in proventricular and gizzard (muscular stomach) epithelia by the proventricular mesenchyme but is suppressed by the gizzard mesenchyme. In the present paper, we studied how the mesenchymes influence this gene expression pattern. For this we produced constructs containing various portions of the 5'-flanking region of the embryonic chick pepsinogen gene,

driving reporter sequences (β -galactosidase or luciferase), and these constructs were transfected into dissociated epithelial cells either from the proventriculus or gizzard. We then recombined these cells with mesenchymal cells and cultured them as cell aggregates. In this way, we were able to dissect the timing and other requirements of the epithelial-mesenchymal interactions for expression of embryonic chick pepsinogen gene. We also report that 1.1 kb of 5'-flanking sequence is sufficient to drive correct expression of embryonic chick pepsinogen gene, although further enhancement was seen if the constructs contained 3.2 kb of upstream sequence.

Key words: chick embryo, pepsinogen gene, stomach-specific expression, epithelial-mesenchymal interaction, cell aggregate culture

INTRODUCTION

One of the most important aims of current developmental biology is to clarify the mechanisms regulating the differential expression of genes during the course of development. The morphological as well as the functional differentiation of an organ necessarily involves the local expression or suppression of sets of genes in the cells constituting the organ. A number of reports on the mechanism of tissue-specific gene expression have been published. Among the genes expressed in the digestive tract and associated organs, 5'-flanking regions have been well characterized in the mouse albumin (Gorski et al., 1986; Pinkert et al., 1987), mouse α 1-antitrypsin (Grayson et al., 1988), rat α -fetoprotein (Jose-Estanyol and Danan, 1988), human sucrase-isomaltase (Wu et al., 1992) and human villin (Robine et al., 1993) genes, by using transgenic mice or by introducing reporter constructs into cell lines. These well-characterized genes are expressed either in the liver or in the intestinal epithelium. There is little information on the function of 5'-flanking region of genes expressed in the stomach.

It is now well established that epithelial-mesenchymal inter-

actions are essential in the spatiotemporally regulated differentiation processes in organogenesis in higher vertebrates (reviewed by Wessells, 1977; Sawyer and Fallon, 1983). The epithelial-mesenchymal interactions in the digestive tract of avian embryos have been studied extensively. The epithelium of the digestive tract is a simple sheet consisting of undifferentiated endodermal cells at early stages and, with the development of the digestive tract, it gradually acquires region-specific morphology and cytodifferentiation under the influence of the underlying mesenchyme (for reviews, see Yasugi and Mizuno, 1990; Yasugi, 1993).

Pepsinogens, zymogens of pepsins, are specifically synthesized in stomach gland cells and have been most extensively studied in relation to epithelial-mesenchymal interactions. Yasugi and Mizuno (1978, 1981, 1982) have investigated the normal development of pepsinogens in chick proventriculus (PV, glandular stomach), and demonstrated that embryonic proventricular epithelial cells synthesize embryo-specific chick pepsinogen (ECPg) which is no longer expressed at the end of embryonic life.

ECPg expression in epithelial cells is induced under the

influence of the proventricular mesenchyme as demonstrated by the epithelial-mesenchymal recombination experiments (Takiguchi et al., 1986, 1988a). For example, when the epithelium of the embryonic gizzard (GZ, muscular stomach) was recombined and cultured with the PV mesenchyme, the epithelium formed PV-like glands and expressed ECPg. However, this PV-specific differentiation was completely inhibited by the GZ mesenchyme even when the PV epithelium was used (Urase and Yasugi, 1993). Subsequently an ECPg cDNA was isolated (Hayashi et al., 1988a) and the induction of ECPg gene expression by the mesenchyme was shown to be regulated at the transcriptional level (Hayashi et al., 1988c). However, we have no information as to the transcriptional regulation of the ECPg gene by mesenchymal signals.

In the present experiments, we studied the regulation of ECPg gene expression in epithelial cells under mesenchymal influences using modified cell aggregate cultures (Townes and Holtfreter, 1955; Moscona, 1961). We introduced recombinant genes consisting of the reporter genes, β -galactosidase and luciferase, connected to the 5'-flanking region of the ECPg gene, into dissociated epithelial cells and these cells were cultivated with various types of mesenchymal cells. We then examined the expression of the reporter genes.

The results obtained demonstrate that, for the induction of transcription of the ECPg gene by PV mesenchymal cells, 1.1 kb 5'-flanking region of ECPg gene is needed. This study represents the first demonstration that mesenchymal signals affect transcription of a gene through its 5'-regulatory region.

MATERIAL AND METHODS

Isolation and dissociation of tissue fragments

The isolation procedure for tissue fragments was the same as that described previously (Takiguchi et al., 1986). In brief, endodermal epithelial fragments and mesenchymal fragments were isolated from the PV and the GZ of 6 day chick embryos (stage 28-29 of Hamburger and Hamilton, 1951) using 0.03% collagenase (CLS, Cooper Biochemical, Malvern, USA) in Tyrode's solution for 1 hour at 37°C. The isolated epithelial and mesenchymal fragments were washed twice with phosphate-buffered saline (PBS) and then treated with trypsin (0.5%, JRH Biosciences, USA) in PBS for 5 minutes at 25°C. After washing with 25% fetal bovine serum in Tyrode's solution, cells were dissociated by gently passing them through a micropipet tip. Cells were washed and pelleted in PBS, and resuspended in Ham's F-12 medium. By this procedure, mesenchymal fragments were completely dissociated into single cells and epithelial fragments became single or very small clusters of cells (up to about 10 cells per cluster). Mesenchymal cells were stored at 4°C until needed.

Construction of luciferase plasmid

Nucleotide sequences from the 3245 bp 5'-flanking region of the ECPg gene were

determined by the Sanger dideoxy method (Sanger et al., 1977) with Taq Dye Primer Cycle Sequence Kit (Applied Biosystems, USA). The vector DNA used for the construction of luciferase vectors was either PGV-C or PGV-B, which contain the luciferase gene with or without simian virus-40 (SV40) promoter-enhancer (Toyo Inki), respectively. The plasmid PGV-3.3 was constructed by inserting 3245 bp from the 5'-flanking region followed by the transcription start site and 38 bp of the first exon of the ECPg gene into *SacI*-*XhoI* sites of PGV-B (Fig. 1). The plasmid PGV-1.1 and the plasmid PGV-73 were constructed by similarly inserting 1021 bp or 35 bp of 5'-flanking region, respectively, followed by the transcription start site and 38 bp of the first exon of the ECPg gene (Fig. 1).

Construction of β -galactosidase plasmid

The plasmid containing bacterial β -galactosidase gene, *pmiwZ*, was kindly provided by Dr Kondoh, Osaka University. *PmiwZ* contains the Rous sarcoma virus long terminal repeat, chick β -actin promoter, the coding sequence of the β -galactosidase gene and the terminal sequence of herpes simplex virus thymidine kinase gene (Suemori et al., 1990; Ueno et al., 1987). The plasmid pCP1.6Z was constructed by fusing 1571 bp of 5'-flanking region followed by the transcription start site and 38 bp of the first exon of the ECPg gene with the coding sequence of the bacterial β -galactosidase gene from pCH110 (Parmacia LKB Biotechnology, Sweden; generous gift of Dr Iba, University of Tokyo).

Transfection of plasmid DNA into epithelial cells

Plasmid DNA (15 μ g) was introduced into epithelial cells of PV and GZ in Ham's F-12 medium supplemented with a transfection-reagent, DOTAP (Boehringer Mannheim Biochemica, Mannheim Germany) (Stamatatos et al., 1988) as indicated by the manufacturer. Cells were

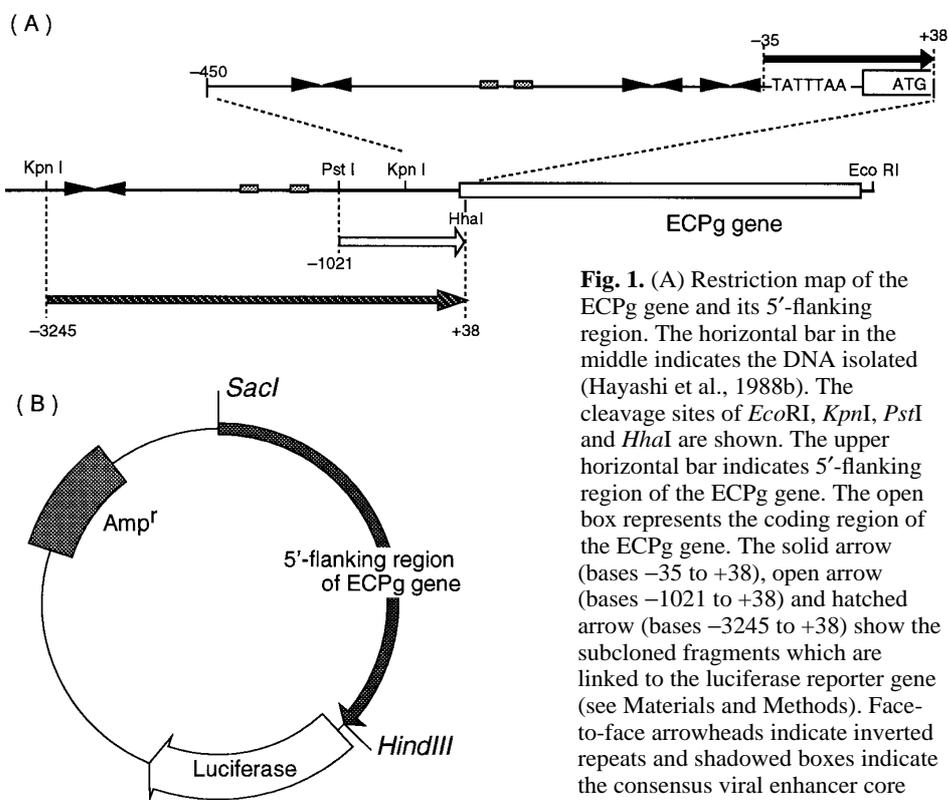


Fig. 1. (A) Restriction map of the ECPg gene and its 5'-flanking region. The horizontal bar in the middle indicates the DNA isolated (Hayashi et al., 1988b). The cleavage sites of *EcoRI*, *KpnI*, *PstI* and *HhaI* are shown. The upper horizontal bar indicates 5'-flanking region of the ECPg gene. The open box represents the coding region of the ECPg gene. The solid arrow (bases -35 to +38), open arrow (bases -1021 to +38) and hatched arrow (bases -3245 to +38) show the subcloned fragments which are linked to the luciferase reporter gene (see Materials and Methods). Face-to-face arrowheads indicate inverted repeats and shadowed boxes indicate the consensus viral enhancer core sequences in positive or negative orientation (see Discussion). (B)

Structure of luciferase constructs. 3.3 kb-73 bp of the 5'-flanking region of the ECPg gene (heavy stippling) are connected with the firefly luciferase gene (open box).

washed with Medium 199 with Earle's salts (GIBCO BRL, USA) 6 hours after transfection.

Cell aggregate culture

Transfected epithelial cells (4×10^5 cells) and mesenchymal cells (8×10^6 cells) were mixed in four combinations, i.e., PV epithelial cells and PV mesenchymal cells, PV epithelial cells and GZ mesenchymal cells, GZ epithelial cells and PV mesenchymal cells, and GZ epithelial cells and GZ mesenchymal cells. These mixtures were centrifuged for 10 minutes at 3000 rpm. The resulting cell aggregates were about 8 μ l in volume. 2 μ l of cell aggregate were placed on porous membranes (Nuclepore SN11049, 8 μ m pore size, Costar Cooperation, Cambridge, UK.), and rested on a stainless-steel grid placed within a small culture dish. Medium was added to reach the filter, so that it moistened the surface of the aggregates but did not cover them. The culture dishes were incubated for 6 days at 37°C in 95% air and 5% CO₂. The culture medium was as described previously (Takiguchi et al., 1988b).

Detection of β -galactosidase activity and ECPg expression

The cultured cell aggregates were fixed with 4% paraformaldehyde in PBS for 30 minutes and treated with β -galactosidase histochemical reaction mixture containing 1 mg/ml 5-bromo-4-chloro-3-indolyl- β -D-garactopyranoside (X-Gal), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 2 mM MgCl₂ in PBS. Incubation was carried out for 4-6 hours at 37°C. The tissues were embedded in paraffin, serially sectioned at 5 μ m, and the sections were used for detection of the ECPg protein by immunohistochemistry. Briefly, deparaffinized sections were treated with monoclonal antibody Y37 (Yasugi et al., 1987) raised against ECPg, then with peroxidase-conjugated goat anti-mouse IgG (Cappel, USA), and were incubated with 3,3'-diaminobenzidine (Sigma Chemical Co., St. Louis, USA) for colour development.

Luciferase assay

The cultured cell aggregates were washed with PBS, sonicated in 25 mM Tris-phosphate (pH 7.5) and centrifuged. Supernatant was added to luminescence-substrate-solution (Toyo Inki, Tokyo, Japan) and luminescence measured with a luminometer. A standard calibration curve was obtained in each experiment using purified luciferase (Sigma).

In situ hybridization

The PV, GZ, small intestine and liver of embryos of day-6, 8, 10 and 15 and a chick 2 weeks after hatching were fixed in 4% paraformaldehyde in PBS for 3 hours at 4°C followed by substitution with 30% sucrose in PBS, embedded in OCT Compound (Miles Inc., USA), and sectioned at 5 μ m in a cryostat. The sections were mounted onto Vectabond-coated slides, treated with Triton X-100 (0.3% in PBS), HCl (0.2 N) and proteinase K (1 μ g/ml in PBS) and post-fixed with 4% paraformaldehyde. The tissue sections were hybridized with digoxigenin (DIG)-labeled RNA probe in 0.3M NaCl, 1 mg/ml *E. coli* tRNA, 10 mM dithiothreitol, 20 mM Tris-HCl (pH. 8.0), 2.5 mM ethylenediaminetetraacetic acid (EDTA), 0.02% ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, 50% formamide and 10% dextran sulfate at 45°C for 16 hours. After brief washing with 2 \times SSC containing 50% formamide, tissue sections were treated with RNase A (20 μ g/ml) at 37°C for 30 minutes, and washed consecutively with 2 \times SSC, 50% formamide at 45°C for 1 hour and with 1 \times SSC, 50% formamide at 45°C for 1 hour.

For immunological detection of hybridized probe, the DIG Nucleic Acid Detection Kit (Boehringer Mannheim Biochemica) was used. Tissue sections were briefly washed in buffer 1 (0.1 M maleic acid, 0.15 M NaCl) and incubated for 30 minutes with 1% blocking reagent in buffer 1. Sections were then incubated with 500-fold diluted anti-DIG-alkaline phosphatase conjugate in the buffer 1 for 30 minutes.

After washing with the buffer 1, tissue sections were incubated with 175 μ g/ml X-phosphate and 337.5 μ g/ml nitroblue tetrazolium salt for 12 hours in the dark. To stop the reaction tissue sections were washed in 10 mM Tris-HCl, containing 1 mM EDTA. Slides were mounted with glycerol and observed under a light microscope.

For preparation of DIG-labeled sense and anti-sense RNA probes, plasmid DNA cBg150, which contains a 10 bp fragment of 3' non-coding region and the poly (A) of the ECPg cDNA (Hayashi et al., 1988b) was subcloned in the *EcoRI* site of pBluescript II SK+ (Stratagene, San Diego, USA). The plasmid DNA was linearized with *PstI* or *HindIII* and transcribed in vitro with T7 or T3 RNA polymerase (Boehringer) following the manufacturer's manual.

RESULTS

ECPg mRNA in normal development of the digestive tract

For the detection of ECPg mRNA, the in situ hybridization technique was used with cBg150 as a probe. ECPg transcripts were detected only in embryonic PV epithelial cells of glandular structures (Fig. 2B-E). No transcripts were detected in other organs (GZ, small intestine and liver, Fig. 2G-I). ECPg mRNA in the proventriculus was detected from in embryos from 8 day's incubation onwards (Fig. 2B) and was most abundant in 15 day embryonic PV (Fig. 2E). Expression of ECPg mRNA completely ceases in the PV of chick 2 weeks after hatching (Fig. 2F). These findings confirmed previous results (Hayashi et al., 1988a) in which the expression of ECPg mRNA was detected by northern hybridization. The present results also demonstrated that expression of the ECPg gene is strictly cell-type-specific and developmental stage-specific at the level of transcription. The localization of ECPg mRNA was the same as that of the ECPg protein which was examined by Yasugi and Mizuno (1981). Therefore we used immunohistochemical methods with Y37 for detection of expression of the ECPg gene in this study.

Inductive ability of dissociated mesenchymal cells and reactive potency of dissociated epithelial cells

For efficient transfection of reporter constructs into epithelial cells and their expression, we used cell aggregate culture method. In this method, dissociated epithelial cells of PV or GZ were mixed with dissociated mesenchymal cells of PV or GZ, centrifuged to make cell aggregates and cultured. During 6 days cultivation, epithelial cells sorted out from mesenchymal cells, so that both epithelial cells and mesenchymal cells were reorganized. When PV epithelial cells were mixed with PV mesenchymal cells, they formed epithelial cysts and gland-like structures, and ECPg was expressed in about 20% of epithelial cells (Fig. 3A). When GZ epithelial cells were mixed with GZ mesenchymal cells, epithelial cells formed only epithelial cysts and never formed gland-like structures, and ECPg expression in the epithelia was not observed (Fig. 3D). PV epithelial cells mixed with GZ mesenchymal cells formed only epithelial cysts and never expressed ECPg just as GZ epithelial cells mixed with GZ mesenchymal cells (Fig. 3C). On the contrary, GZ epithelial cells mixed with PV mesenchymal cells formed cysts and gland-like structures and expressed ECPg (Fig. 3B). Thus, epithelial cells retained the ability to respond to the inductive influences of PV and GZ mesenchymes and mesenchymal cells retained inductive

ability according to their origins as in the *in vitro* organotypic culture (Takiguchi et al., 1988a). Hence, cell aggregate culture was judged to be suitable for the DNA transfection assay.

Expression of the luciferase reporter gene driven by the 5'-flanking region of the ECPg gene

To examine the transfection efficiency of the construct, the plasmid *pmiwZ*, containing β -actin promoter linked to β -galactosidase sequence, was introduced into dissociated epithelial cells by lipofection. The number of cells that incorporated and expressed the reporter construct was counted 1 day after transfection. For both PV and GZ epithelial cells, about 3% expressed β -galactosidase and there was no difference in transfection efficiency between the two cell types (data not shown).

PGV-3.3, which contains 3.2 kb of 5'-flanking region of the ECPg gene and the luciferase reporter gene, was introduced into dissociated PV or GZ epithelial cells, the cells cultivated with PV or GZ mesenchymal cells in the cell aggregate culture and transient expression of luciferase activity measured. Plasmid PGV-C, which contains the SV40 enhancer-promoter and the luciferase reporter gene, was transfected as a positive control. Luciferase activities were not significantly different among four types of aggregate in which PGV-C was introduced, so these values were used to normalize the expression of luciferase in epithelial cells. The luciferase activities in the cell aggregates transfected with promoterless plasmid, PGV-B, were as low as those in non-transfected cell aggregates and we regarded them as background level.

The results in Fig. 4, in which luciferase activities are expressed as a percentage of the positive controls of each experiment, showed that the expression of luciferase activity in epithelial cells transfected with PGV-3.3 is regulated by mesenchymal cells. Thus, in the aggregates that contained PV

epithelial cells and PV mesenchymal cells, high luciferase activity was observed (13.3 ± 1.2). In contrast, only the basal level of luciferase activity was detected in aggregates of GZ epithelial cells and GZ mesenchymal cells (0.7 ± 0.3). Thus the 3.2 kb 5'-flanking sequence appears to be transcriptionally

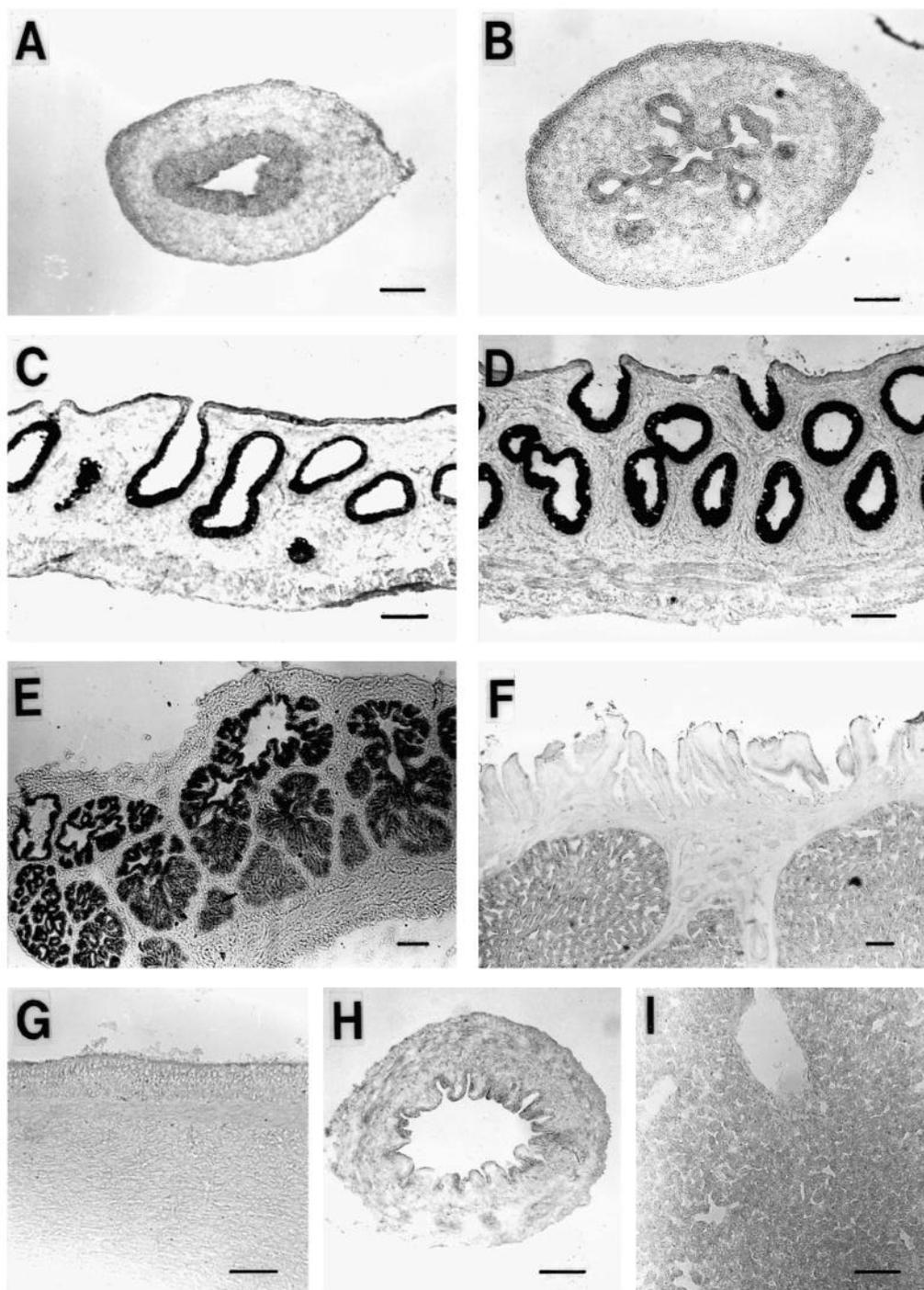


Fig. 2. Detection of ECPg transcripts in normal chick tissues by *in situ* hybridization with anti-sense probe for ECPg mRNA. PV of 6-day embryo (A); PV of 8-day embryo (B); PV of 10-day embryo (C); PV of 12-day embryo (D); PV of 15-day embryo (E); PV of 2-week chick (F); GZ of 15-day embryo (G); small intestine of 15-day embryo (H); liver of 15-day embryo (I). ECPg transcripts were detected only in the embryonic PV epithelium from 8 days and were most abundant in the 15-day embryo. Scale bar, 100 μ m.

competent to direct expression of the luciferase gene only in ECPg-expressing epithelial cells. When PV epithelial cells with PGV-3.3 were mixed with GZ mesenchymal cells luciferase activity was low (2.83 ± 1.5). On the other hand, when GZ epithelial cells containing PGV-3.3 were mixed with PV mesenchymal cells, the activity (11.8 ± 1.5) was almost as high as that in the aggregates of PV epithelial cells and PV mesenchymal cells. These results unequivocally demonstrate that the 3.2 kb 5'-flanking sequence can mediate influences of PV and GZ mesenchymes, respectively, in the heterologous cell aggregate cultures.

Next, two shorter fragments of the 5'-flanking region of the ECPg gene were linked to the luciferase gene and introduced into PV and GZ epithelial cells. Transfection of a construct containing bases -1021 to +38 (PGV-1.1) yielded activity 20-30% lower than that obtained with PGV-3.3 in PV or GZ epithelial cells cultivated with PV mesenchymal cells (in PV epithelial cells, 9.1 ± 0.8 ; in GZ epithelial cells, 9.3 ± 0.3), whereas only basal activities were obtained when these epithelial cells were mixed and cultivated with GZ mesenchymal cells (in PV epithelial cells, 2.0 ± 0.7 ; in GZ epithelial cells, 1.4 ± 0.2). Therefore, the 1.0 kb 5'-flanking sequence is also sufficient for transcription of the ECPg gene in an organ-specific manner and for receiving the signals from mesenchymal cells, but the activity is slightly lower.

When a construct containing bases -35 to +38 (PGV-73) was used, luciferase activity was only at background level in all combinations.

To examine the possibility that the results with PGV-3.3 and PGV-1.1 were due to enhanced proliferation of epithelial cells induced by culturing them with PV mesenchymal cells, or to epithelial cell death caused by GZ mesenchymal cells, the plasmid pmiwZ was introduced into 1×10^4 epithelial cells and, after 6 days' cultivation, we counted the β -galactosidase-positive cells in each aggregate. The number of the β -galactosidase-positive cells was about 800 per aggregate and there was no significant difference between four types of aggregates (data not shown). Therefore the difference in luciferase activity between aggregates is not due to differences in proliferation rates of the epithelial cells.

Cell-type-specific expression of the β -galactosidase gene driven by 5'-flanking region of ECPg gene

To ascertain that reporter gene expression controlled by the 5'-flanking region of the ECPg gene is limited to epithelial cells that normally express ECPg, the plasmid pCP1.6Z, containing 1571 bp of 5' flanking region and 38 bp of the first exon of the ECPg gene linked to the β -galactosidase gene, was introduced into PV or GZ epithelial cells and cultured with PV or GZ mesenchymal cells. In aggregates composed of PV or GZ epithelial cells and PV mesenchymal cells, some epithelial cells expressed β -galactosidase whereas, in aggregates of PV or GZ epithelial cells and GZ mesenchymal cells, β -galactosidase was not detected. The aggregates in which β -galactosidase was detected were subjected to immunohistochemistry with Y37 to determine whether β -galactosidase staining was strictly restricted to ECPg-expressing epithelial cells. Fig. 5 shows that β -galactosidase-expressing cells also express the ECPg protein. This indicates that 1.6 kb of 5'-flanking sequence may be sufficient for the cell-type-specific transcription of ECPg gene in epithelial cells induced by PV mesenchymal cells.

Luciferase activity was as low as background level when PGV-1.1 was transfected into PV or GZ mesenchymal cells and they were recombined with PV or GZ epithelial cells.

DISCUSSION

Establishment of cell aggregate culture for transfection and expression of foreign DNA in epithelial cells

In this study, we modified cell aggregate culture method, in which both epithelium and mesenchyme were dissociated into small clusters of cells, mixed and cultivated. In this culture method, various plasmids were efficiently introduced into epithelial cells. For dissociated epithelial cells the transfection efficiency was 20- to 30-fold higher than in the case of intact epithelial sheets (data not shown). With this method, we could detect expression of the luciferase reporter gene under the control of 5'-flanking sequence of the ECPg gene for the first time. We also showed that PV and GZ epithelial cells retain the ability to undergo morphogenesis and cytodifferentiation under the conditions of this culture. Their differentiation into PV-like epithelial cells as evidenced by gland formation and ECPg expression is properly induced by PV mesenchymal cells and suppressed by GZ mesenchymal cells even if the normal organization of these epithelia and mesenchymes has been destroyed.

There have been many reports on organ culture systems of embryonic organs such as mammary gland (Kratochwil, 1969; Sakakura, 1987), tooth (Kollar and Braid, 1970; Mina and Kollar, 1987; Snead et al., 1984), kidney (Saxén et al., 1968; Ekblom et al., 1981), digestive tract (Yasugi, 1993) and submandibular gland (Nogawa and Nakanishi, 1987; Oster et al., 1983). These reports, in which intact organs or tissue recombinants of isolated epithelium and heterotypic or homotypic mesenchyme were cultivated, have contributed to elucidate the mechanisms of epithelial-mesenchymal interactions in the organogenesis of these organs. To attain a better understanding of epithelial-mesenchymal interactions at the molecular level, however, it is necessary to establish a culture system that allows easier application of molecular biological techniques. Takiguchi et al. (1988b) and Urase and Yasugi (1993) reported a method to dissociate and reaggregate mesenchymal cells and to recombine them with intact epithelium. They demonstrated that PV and GZ mesenchymal cells retain the influences on morphogenesis and cytodifferentiation of the PV or GZ epithelium even when the normal organization of the mesenchyme had been destroyed. Although this method is useful for the analysis of mesenchymal influences, it was difficult to introduce reporter constructs into the reacting epithelial sheet.

The 5'-flanking region is necessary for expression of the ECPg gene

The present study revealed the structure and function of the 5'-flanking region of ECPg gene, which is expressed specifically in the glandular stomach.

Transfection of chimeric genes containing the 5'-flanking region of the ECPg gene into epithelial cells suggested that there is an organ-specific regulatory region within 3245 bases of the start site of transcription. Within this flanking region,

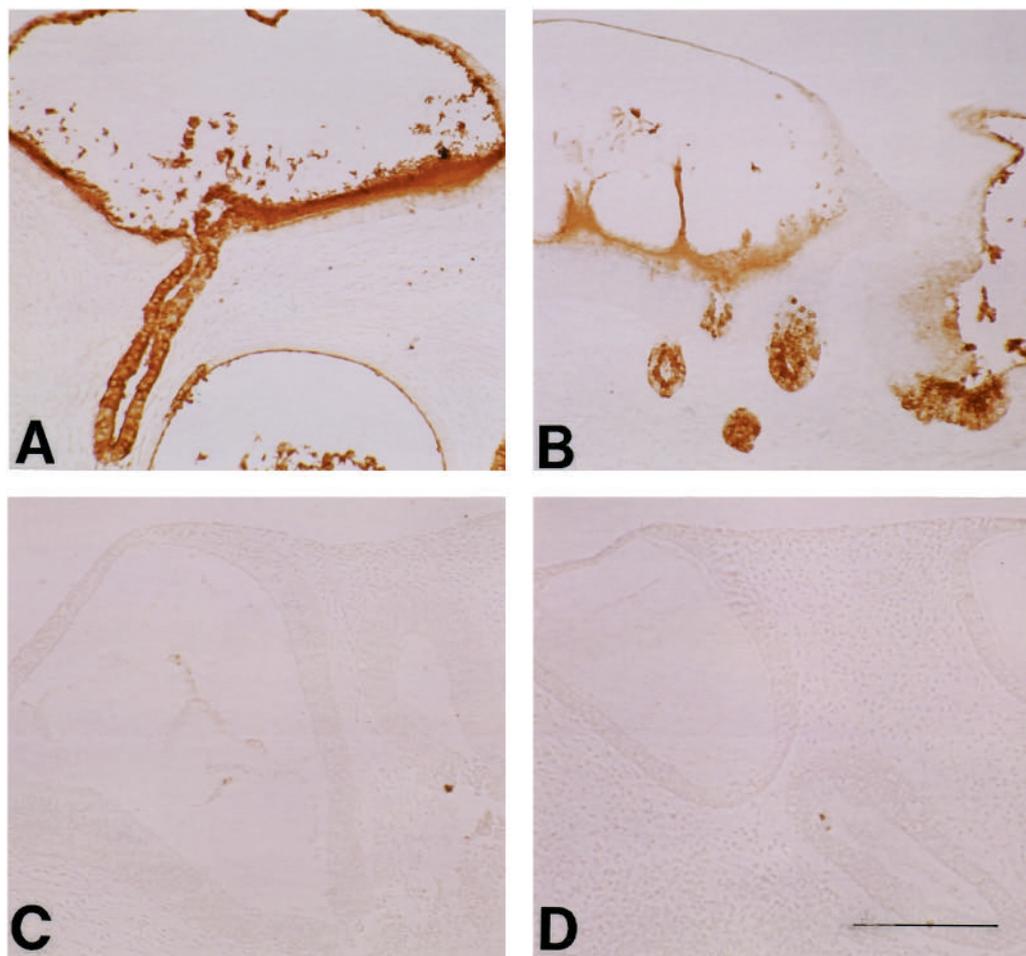


Fig. 3. The structures formed by epithelial cells and ECPg expression in the aggregates cultured as cell aggregates. The aggregates were sectioned and subjected to detection of the ECPg protein by antibody Y37. In the aggregates containing PV (A) and GZ (B) epithelial cells and PV mesenchymal cells, epithelial cells formed cysts and gland-like structures and expressed ECPg. In the aggregate containing PV (C) and GZ (D) epithelial cells and GZ mesenchymal cells, epithelial cells formed only epithelial cysts and never formed gland-like structures and ECPg expression could not be detected. Scale bar, 100 μ m.

1021 bases from the start site supported the cell type-specific transcription of ECPg gene. There is marked enhancement of transcription in epithelial cells transfected with PGV-3.3 which contains bases -1021 to -3245 in addition to bases -1021 to $+38$, although we don't know whether this region between -1021 and -3245 is involved in cell-type-specific transcription of ECPg gene or simply enhances its transcription.

In the region from -1021 to -3245 , there are two sequences, CTTACCA and CAAACCA, 1853 bp and 1403 bp upstream from the start site, respectively, which are identical to the inverted consensus viral enhancer core sequence (Weiher et al., 1983), C(A,T)(A,T)(A,T)CCA (Fig. 1). It is noted that there are two other sequences identical to this sequence or to the inverted sequence of this at 272 bp and 361 bp upstream from the start site (Hayashi et al., 1988b). In the region within 1021 bases of the start site of transcription, there are three inverted repeats consisting of 8 or 9 bases with one mismatch (Fig. 1). Whether the sequences described above are involved in ECPg expression is open to question at present.

Recently Lorenz and Gordon (1993) demonstrated that 1035 bp of the 5'-flanking region of the H^+/K^+ -ATPase β -subunit gene (Canfield and Levenson, 1991) and 1029 bp of the 5'-flanking region of the intrinsic factor gene are necessary for their specific transcription in parietal cells, which produce H^+ . In these regions, there are two or three enhancer core sequences as described above. Moreover, the 5'-flanking region of human

(Hayano et al., 1988) and rat (Ishihara et al., 1988) pepsinogen genes which are expressed specifically in zymogenic (chief) cells in the stomach contain the same sequences. Chick proventricular gland epithelial cells are equivalent to parietal cells and zymogenic cells in mammalian stomach, because both HCl and pepsinogen are produced in the same cells in the proventriculus (Toner, 1963). These results favour the inference that the enhancer core sequences are involved in the cell-type-specific transcription of genes expressed in the stomach.

In mammals, prochymosin production in the gastric mucosa is replaced by pepsinogen production during the first few weeks of the neonatal period (Foltmann et al., 1981). Expression of the prochymosin gene is thus similar to ECPg gene expression which occurs transiently during the embryonic period and is replaced by expression of adult chick pepsinogen. So one might expect some homology between the 5'-flanking sequences of the prochymosin gene and the ECPg gene. However, when the 5'-flanking sequence of ECPg gene is compared with that of the calf prochymosin gene (Hidaka et al., 1986), no significant similarities are observed, and there is no enhancer core sequence in 200 bp of the 5'-flanking sequence of the calf prochymosin gene. The enhancer core sequences may exist in a more distant 5' region of the gene or there may be some other regulatory sequences in the genes expressed in the mammalian stomach.

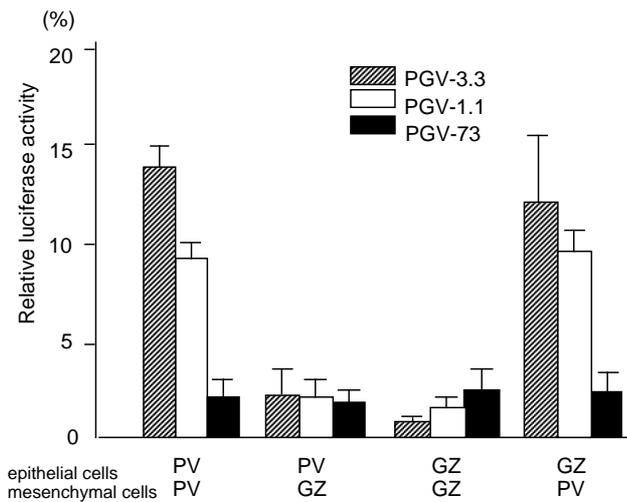


Fig. 4. Activation and repression of transcription of the luciferase reporter gene under the influence of PV or GZ mesenchymal cells. Plasmids containing the luciferase gene under the control of various fragments of the 5'-flanking region of the ECPg gene were transfected into PV or GZ epithelial cells, and after cultivation with PV or GZ mesenchymal cells luciferase activity was measured in cell extracts. Hatched bars indicate luciferase activities in cells transfected with PGV-3.3 which contains 3245 bp of 5'-flanking region of the ECPg gene. Open bars indicate luciferase activities in cells transfected with PGV-1.1, which contains 1021 bp of the 5'-flanking region of the ECPg gene. Solid bars indicate luciferase activities in the cells transfected with PGV-73, which contains 35 bp of the 5'-flanking region of the ECPg gene. All values are averages of three determinations and are expressed as percentages of luciferase activity in control cells transfected with the PGV-C plasmid, which contains enhancer and promoter sequences from SV40.

Previously we investigated the methylation state of DNA of the 5'-flanking region of the ECPg gene isolated from ECPg-expressing and non-expressing organs and found no significant difference in the degree of methylation (Fukuda et al., 1994). We suppose, therefore, that the methylation state of the 5'-flanking region itself is not involved in the regulation of organ-specific expression of the ECPg gene, but some factors that bind to this region are important to modulate its activity.

Mesenchymal influences on ECPg expression are mediated by the 5'-flanking region

Recently some mesenchymal factors involved in epithelial-mesenchymal interactions have been identified, such as epimorphin (Hirai et al., 1992), TGF- β (Heine et al., 1987) and hepatocyte growth factor (Montesano et al., 1992; Sonnenberg et al., 1993). Although these factors may play important roles in the morphogenesis and cytodifferentiation of epithelia in general, we suppose that the mesenchyme of each organ such as PV and GZ emits more specific factors which evoke organ-specific reaction of the epithelium. The present paper shows that such mesenchymal signals affect the transcriptional activity of the ECPg gene through its 5'-flanking region though how this is activated still remains a question.

There is evidence that some components of the basement membrane are important for the expression of epithelial cell-

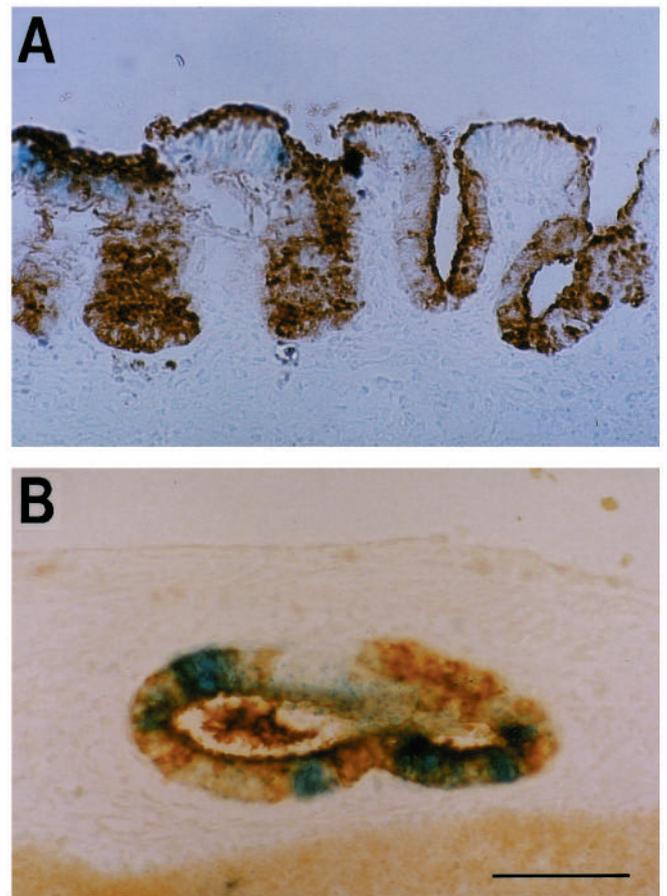


Fig. 5. Expression of β -galactosidase activity in epithelial cells cultured as cell aggregates. The plasmid, pCP1.6Z containing 1.6 kb of the 5'-flanking region of the ECPg gene linked to the coding sequence of the β -galactosidase gene, was introduced into PV epithelial cells (A) and GZ epithelial cells (B) and cultivated with PV mesenchymal cells for 6 days in cell aggregate culture. Blue staining and brown staining show β -galactosidase activity and ECPg protein, respectively. In both types of aggregate, β -galactosidase-positive cells also express the ECPg protein. The weak brown staining in the lower part of B is non-specific staining of the filter. Scale bar, 50 μ m.

specific genes, such as β -casein in the mammary gland (Schmidhauser et al., 1992, Streuli et al., 1991) and albumin in the liver (Zaret et al., 1990). For the induction of ECPg, contact of the PV mesenchyme with epithelial cells over a rather wide range is necessary (Takiguchi-Hayashi and Yasugi, 1990). It is then possible that some components of the basement membrane are involved in the mediating these inductive interaction.

The ECPg gene is one of the structural genes that are expressed after gland formation (day 6 of incubation) in the PV epithelium. This fact suggests that there are several steps between the time at which epithelial cells receive the mesenchymal signals and the onset of transcription of the ECPg gene. Thus, PV epithelial cells which receive the PV mesenchymal signals are committed to differentiate into PV epithelial cells and soon produce *trans*-regulatory factors, which activate transcription of the ECPg gene by binding to a regulatory sequence 1.1 kb from the transcription start site. The

gizzard mesenchyme, in contrast, emits some inhibitory factors to suppress the production of the *trans*-regulatory factors activating the transcription of the ECPg gene in epithelial cells. Thus, although the gizzard epithelium has the potential to express the ECPg gene (Takiguchi et al., 1986), it does not express ECPg in normal development.

Nuclear factors extracted from PV but not from liver were found to interact with the 5'-flanking region (-398 to -195) of the ECPg gene (Fukuda and Yasugi, unpublished data). The region to which these factors bind contains the consensus enhancer core sequence or its inverted sequence located at -361 bp and -271 bp. This suggests that the putative *trans*-acting factors are produced in the PV epithelium and these factors bind to *cis*-elements of the ECPg gene to enhance transcriptional activity of the gene. To examine whether the expression of these *trans*-acting factors is modulated by the influence of the mesenchyme is very important in understanding the molecular mechanisms of tissue interactions. The purification of the factors is now carried out in our laboratories.

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