

Regulation of pepsinogen gene expression in epithelial cells of vertebrate stomach during development

SADAO YASUGI*

Department of Biology, Faculty of Science, Tokyo Metropolitan University, Tokyo, Japan

ABSTRACT Pepsinogens are zymogens of pepsins, aspartic proteases working as digestive enzymes in the vertebrate stomach, of which biological and molecular properties have been extensively studied. In developmental biology, pepsinogens offer excellent molecular markers of differentiation of stomach epithelial cells, since their expression is strictly limited to those cells and there are some isozymes that are expressed in developmental stage-specific manner. It is now well established that the expression of embryonic chicken pepsinogen (ECPg) gene is regulated by epithelial-mesenchymal interactions: it is mesenchyme that determines the expression pattern of ECPg along the digestive tract, by supporting or inhibiting the intrinsically endowed ability of epithelial cells to express it. In the present review article, I will describe recent molecular biological and experimental embryological consequences of our studies on the regulation of ECPg expression by mesenchymal cells, with special attention to the nature of mesenchymal factors and the molecular mechanisms of reactivity of epithelial cells to the mesenchymal influences.

KEY WORDS: *pepsinogen genes, stomach epithelium, tissue interactions, ontogeny, phylogeny*

Introduction

One of the most important problems in developmental biology today is to elucidate the mechanisms regulating differential gene expression during development: characteristic features of each stage of animal development are determined by the set of genes transcribed at that moment, and the analysis of regulatory mechanisms underlying the developmental stage-specific expression of genes is vital to understand the whole sequences of developmental phenomena in animals.

Although there have been many studies concerning differential gene expression in development, the interpretation of the phenomena is far from completion. This is partly because there are only a few good systems for the analysis of mechanisms of differential gene expression.

In this review article, I will describe the study with pepsinogen gene expression during development, with special emphasis on regulation by tissue interactions. Pepsinogen gene of chicken embryos is a very useful gene to analyze the differential expression during ontogeny, since it is expressed strictly in a cell population in a definite developmental stage, and since it is transcribed in a large quantity.

Biology and biochemistry of pepsinogens

Pepsinogen and pepsin

Pepsinogens are zymogens of pepsins, digestive enzymes

working in the stomach of vertebrates at low pH ranges (pH 2-3). Pepsinogen is produced and secreted by epithelial cells of the stomach, and cleaved at the appropriate site by autocatalytic action under the acidic conditions of gastric fluid to generate active pepsin molecules.

Pepsin is one of the best studied digestive enzymes because of its abundance in gastric juice and was investigated as early as in the first half of nineteenth century by Schwann. Northrop (1930) crystallized bovine pepsin, and it was the first example of crystallization of digestive enzymes. Thereafter the structure and mode of action of pepsin have been the subjects of an enormous number of studies (Vonk and Western, 1984). Recently, the molecular mechanisms of regulation of pepsinogen gene expression have also been investigated (see below).

Structure of pepsinogen polypeptides

To date more than 50 species of pepsinogens from more than 30 vertebrate species have been purified and their amino acid sequences determined. Although there are differences among molecular weights of these pepsinogens, the majority of pepsinogens have leader sequences of about 15 amino acids, activation peptides of about 30 amino acids and active pepsins of

Abbreviations used in this paper: PV, proventriculus; GZ, gizzard; ECPg, embryonic chicken pepsinogen; APg, adult pepsinogen.

*Address for reprints: Department of Biology, Faculty of Science, Tokyo Metropolitan University, 1-1, Minamiosawa, Hachioji, Tokyo 192-03, Japan. FAX: 426-77-2559.

0214-6282/94/\$03.00

© UBC Press
Printed in Spain

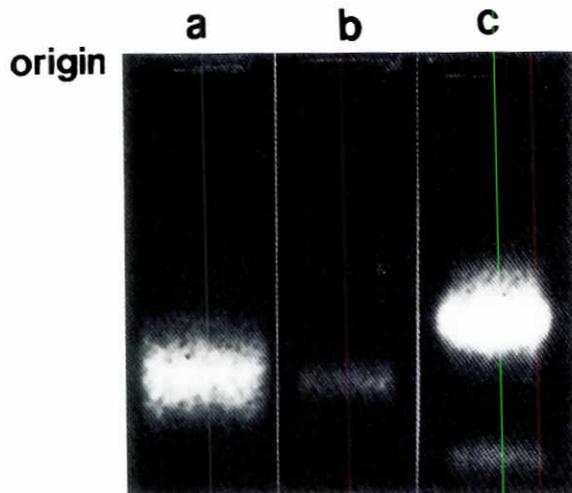


Fig. 1. Zymograms of (a) crude extract of 15-day embryonic PV, (b) purified ECPg and (c) crude extract of adult PV. Samples were electrophoresed on polyacrylamide gel and pepsinogens were visualized as white bands (from Yasugi and Mizuno, 1981b).

about 340 amino acids. The active site contains two aspartic acids and hence pepsin is classified as one of the aspartic proteases.

Pepsinogen-producing cells

Pepsinogen is produced in the epithelial cells of the stomach in the vertebrates, the only exception being the one produced in the esophageal gland cells of the bullfrog (Yakabe *et al.*, 1991). Pepsinogen-producing cells in the mammalian stomach are called chief cells and are distinguished from acid-producing parietal cells and mucus-secreting cells. In the lower vertebrates, however, stomach gland cells are said to produce both pepsinogens and acid at the same time (Toner, 1963). Pepsinogen-producing cells are characterized by the presence of zymogen granules and well-developed Golgi apparatus. Recently the presence of pepsinogen in these cells has been shown by the immunohistochemical methods, both light microscopical and electron microscopical. These studies unequivocally demonstrate that pepsinogens are produced exclusively in the epithelial cells of the stomach and that pepsinogens can be used as very specific markers of the differentiation of these cells.

Ontogeny and phylogeny of pepsinogen expression

Changes in isozyme patterns of pepsinogen during development

Stomach epithelial cells of the vertebrates produce several species of pepsinogens which differ in amino acid compositions and peptic activities. It is also evident in several animal species that the electrophoretic patterns of these isozymes change during development. The typical example of this is shown in the avian stomach.

The avian stomach is subdivided into the proventriculus (PV), the glandular stomach, and the gizzard (GZ), the muscular stomach, and pepsinogens are produced exclusively in the former. When the extracts of PV from various developmental stages of

chicken embryos and hatched chicken are subjected to native polyacrylamide gel electrophoresis and the bands of pepsinogens are visualized by immersing the gel in the hemoglobin solution as substrate and staining of hemoglobin after its digestion in acidic solution (zymogram), it is revealed that the embryonic stomach has isozymes different from the adult ones (Yasugi and Mizuno, 1981a; Fig. 1). We purified the embryonic chicken pepsinogen (ECPg) and found that it is a unique pepsinogen with rather high molecular weight (56,000 as pepsinogen and 53,000 as pepsin; Yasugi and Mizuno, 1981b). The transition from ECPg to adult type of pepsinogen occurs in the last several days of incubation, because ECPg ceases to be expressed at about 18 days of incubation and adult type of pepsinogens begins to appear within one or two days after hatching.

The changes of isozyme species during development have been observed also in other animal species. For example, human fetal stomach expresses pepsinogen IV (fetal pepsinogen) while normal adult stomach also expresses other pepsinogens such as pepsinogen I, II and III (Hirsch-Marie *et al.*, 1976). In the mouse and rat, the changes of isozyme patterns in fetal and post-natal periods have been well studied and the involvement of some hormones such as hydrocortisone is suggested (Furihata *et al.*, 1972; Muto and Tani, 1979; Yasugi *et al.*, 1987). Developmental change of pepsinogen isozymes has also been reported in the rabbit (Kageyama *et al.*, 1990).

Phylogenetic relationship of pepsinogens in vertebrates

As is mentioned above, pepsinogens of various vertebrate species have been isolated and characterized so far. From these data we can construct the phylogenetic trees of pepsinogen species of vertebrates. We distinguish 3 types of pepsinogens, namely, pepsinogen A, progastricsin and prochymosin, according to their amino acid sequences and enzymatic properties. Pepsinogen A is major pepsinogen in the adult animals whereas prochymosin is expressed predominantly in the stomach of young animals, at least in mammals. Interestingly enough, ECPg belongs to prochymosin group rather than pepsinogen A group to which chicken pepsinogen A belongs (Hayashi *et al.*, 1988a). Thus prochymosin group is thought to be a special type of pepsinogen which is expressed in the stomach of embryonic and young stages in development.

We also purified adult chicken and quail pepsinogens (APg) and obtained antibodies to these pepsinogens and to ECPg (Yasugi *et al.*, 1987). With these antibodies as probes, we surveyed the expression of two types of pepsinogens in the adult stomach epithelium of various vertebrates (Yasugi, 1987). The results indicated that, in all species examined, stomachs express anti-APg-reactive materials whereas only stomachs of adult bony fishes and elasmobranchia possess anti-ECPg antibody-reactive materials (Yasugi *et al.*, 1988). These results suggest that the expression of pepsinogen species during ontogeny of each vertebrate species may reflect the evolutionary relationships of pepsinogens.

The most intriguing fact in studying the phylogeny of pepsinogens is that the ascidians have anti-ECPg antibody-reactive substance in their stomach and intestine (Yasugi *et al.*, 1989b). The substance is detected by immunohistochemical method but not by Western blotting method. Moreover the stomachs of ascidians showed very low, if any, activity of protease at low pH conditions. So it is difficult to conceive that the substance immunoreactive to the antibody works as a digestive enzyme. We can postulate that

the substance has a different function in the digestive tract of ascidians, for example as mucus. The cloning of a gene coding this substance in ascidians will elucidate more precisely the phylogenetic relationship of this substance to vertebrate pepsinogens and, at the same time, the mode of regulation of expression of pepsinogen genes during ontogeny and phylogeny.

Molecular biology of pepsinogen genes and their expression

Pepsinogen genes of vertebrates

Recently pepsinogen genes of various animal species including human have been cloned. The first report was the cloning of cDNA of swine pepsinogen by Sogawa *et al.* (1981). The first human pepsinogen gene was cloned soon after that (Sogawa *et al.*, 1983). Other pepsinogen genes and cDNAs so far cloned include calf prochymosin (Hidaka *et al.*, 1986), human pepsinogen C (Hayano *et al.*, 1988; Taggart *et al.*, 1989), monkey pepsinogen A (Evers *et al.*, 1988), rat pepsinogen C (Ishihara *et al.*, 1989), bovine chymosin (Moir *et al.*, 1982), rabbit pepsinogen (Kageyama *et al.*, 1990) and ECPg (Hayashi *et al.*, 1988a,b). Pepsinogen genes of mammals and bird have, without exception, 9 exons interrupted by the introns of various lengths (Hayashi *et al.*, 1988b, Fig. 2A). This suggests that these genes evolved from a common ancestral gene. The base sequence around the active site aspartic acids is well conserved in these genes. The fact that two aspartic residues of active site are present in the different exons indicates that pepsinogen genes have evolved from a single ancestral exon.

Regulatory element of pepsinogen genes

In the course of cloning of pepsinogen genes in various animal species, 5' regulatory regions were also analyzed. Several interesting elements were reported to exist in these regions. In the gene of rat pepsinogen C, for example, there is an Sp 1 binding site core sequence (GC box=GGGCGG) which is thought to be involved in the transcriptional regulation of this gene (Ishihara *et al.*, 1989). The nucleotide sequence of the 5' region of the rat pepsinogen C gene is similar to that of human pepsinogen C gene (Sogawa *et al.*, 1983), but the latter has no Sp 1 binding site.

Ishihara *et al.* (1989) showed that the production of the rat pepsinogen C increases during development, and hydrocortisone has an enhancing effect on the level of transcription of this gene *in vivo*. They also demonstrated, by Southwestern blotting method, that a 25-kDa protein obtained from the gastric mucosa binds specifically to 5' region of the gene. It is especially important that this nuclear fraction does not exist in the liver.

In our laboratory, the transcription factors of ECPg gene have been investigated by gel retardation assay. The 5' region of ECPg gene has several candidate sequences for the binding of *trans*-acting regulatory factors such as 9 nucleotides' inverted repeat at 73-42 bp upstream from the cap site and two sequences identical with the consensus viral enhancer core sequences at 272 and 361 bp upstream from the cap site, respectively (Hayashi *et al.*, 1988b). We used three segments of 5' region of ECPg gene (398-285, 285-195, 192-130 bp upstream from the cap site, respectively) as probes for gel retardation assay. Results showed that probe 285-195 specifically bound to some nuclear factors obtained from PV and formed a shift band but it did not bind to the nuclear extract from the liver to form the same shift band (Fukuda *et al.*, unpublished results). At present the nuclear factor from PV has not been

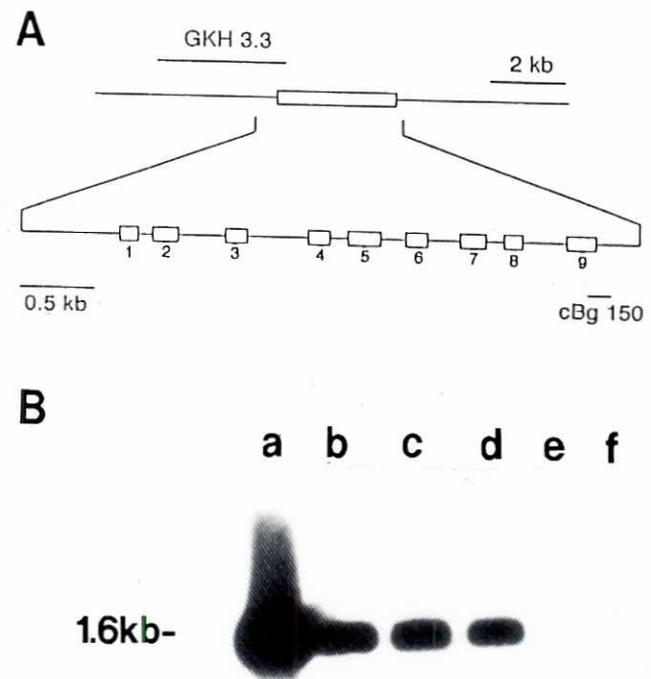


Fig. 2. Structure of ECPg gene and its expression in tissue recombinants. (A) Structure of isolated ECPg gene (from Hayashi *et al.*, 1988b). Upper horizontal bar indicates the isolated DNA and open box the transcribed region of ECPg gene. Lower bar shows the detail of transcribed region and open boxes indicate the nine exons. GKH 3.3 is the regulatory region used to make a construct with luciferase gene (see Fig. 3). cBg 150 is a probe used for Northern analysis shown below. (B) Northern analysis of mRNAs extracted from (a) normal 15-day PV and from recombinations of PV mesenchyme and the epithelium of (b) esophagus, (c) PV, (d) GZ, (e) small intestine or (f) allantois (from Hayashi *et al.*, 1988c).

purified, and it is interesting from the view point of regulation of ECPg gene expression to test the binding of nuclear factors from adult and embryonic PV.

DNA methylation and expression of pepsinogen genes

Tissue (organ)-specific and developmental stage-specific expression of certain genes is known to be regulated by the degree of methylation of bases, especially cytosine bases next to guanine bases (CG), in DNA of the relevant genes (Cedar and Razin, 1990). Recent studies demonstrated that the expression of pepsinogen genes in the rat (Ichinose *et al.*, 1988a) and in the human (Ichinose *et al.*, 1988b) are hypomethylated in the organ in which these genes are activated and transcribed. They showed that the demethylation of the genes occurred a little later than the onset of transcription of the genes and argued that the demethylation itself does not play a causal role in gene activation but is important for the stabilization of gene expression.

They further revealed, by an elaborate technique in which pepsinogen-producing cells of the guinea pig were isolated by cell elutriation, that methylation of pepsinogen gene occurs only in this cell type (Ichinose *et al.*, 1988c).

We analyzed the methylation of ECPg during chicken development in various organs. As mentioned above, ECPg gene is transcribed in the epithelial cells of PV from day 9 to 18 of

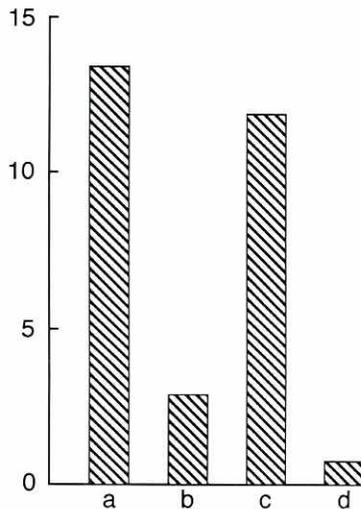


Fig. 3. Luciferase activities (arbitrary units) of cell aggregates composed of (a) PV epithelial cells and PV mesenchymal cells, (b) PV epithelial cells and GZ mesenchymal cells, (c) GZ epithelial cells and PV mesenchymal cells and (d) GZ epithelial cells and GZ mesenchymal cells. Epithelial cells were transfected by lipofection with construct composed of 5' regulatory region of ECPg gene (GKH 3.3, see Fig. 2) and luciferase gene, recombined with mesenchymal cells and cultivated *in vitro* for 3 days (Fukuda *et al.*, unpublished results).

incubation. We then extracted high molecular weight DNA from PV, GZ, small intestine and liver of day 8 and 15 of embryonic period and of hatched chicken. DNA samples were digested with methylation-sensitive restriction enzymes, *Hha* I or *Hpa* II, or by *Msp* I, methylation-insensitive isoschizomer of *Hpa* II, and analyzed by Southern hybridization with a genomic DNA fragment of ECPg as a probe. The results indicated that the degree of methylation detected by *Hpa* II was lower in PV after the onset of ECPg expression, as in the case of the rat and human. The unexpected finding was that the hypomethylated state of ECPg gene was maintained even in the post-hatch period when the expression of ECPg gene completely ceases. Moreover, when *Hha* I was used as restriction enzyme, the degree of methylation augmented in all organs tested except PV (Fukuda *et al.*, 1994). These results suggest that methylation of ECPg, at least of sequences recognized with *Hpa* II (CCGG) and *Hha* I (GCGC), is involved in the regulation of organ-specific expression of ECPg, but developmental stage-specific regulation is brought about by different mechanisms.

Regulation of pepsinogen gene expression by tissue interactions

Effect of various mesenchymes on the expression of ECPg in the epithelium

Tissue interactions play very important roles in the morphogenesis and cytodifferentiation of many organ systems. There is ample evidence showing the importance of the mesenchymal tissues in the determination of epithelial cells during organogenesis (for review see Mizuno and Yasugi, 1990). We have carried out many experiments on the epithelial-mesenchymal interactions involved in the organogenesis of digestive organs in the chicken embryos (Yasugi and Mizuno, 1990; Yasugi, 1993).

When we analyze the tissue interactions of organogenesis of various organ systems, it is prerequisite to know the precise mode of action of one tissue on the other. Since the digestive organs consist of the epithelial and mesenchymal tissues, our first analyses were to survey the influences of the mesenchymes derived from various organs on the expression of ECPg in the epithelium. We took esophagus, PV, GZ, and small intestine of 6-day chicken

embryos and the allantois of 3-4-day embryos, and separated the epithelium from the mesenchyme by appropriate proteases. The separated tissues were recombined and cultivated organotypically. After several days' cultivation the recombinants were recovered and the expression of ECPg, as well as their morphological differentiation, were examined immunohistochemically or using cDNA of ECPg.

The methods of cultivation were diverse: the *in vivo* methods such as transplantation onto chorioallantoic membrane or into the coelomic cavity, or the *in vitro* methods such as those of Wolff and Haffen (1952) or Trowell (1954). Often more than two methods of cultivation were adopted in one type of experiment to avoid the possible effect, quantitative or qualitative, of the cultivation method on the results.

When the epithelia of esophagus, PV and GZ were recombined and cultivated with the mesenchyme of PV, these epithelia definitively formed complex glands and gland epithelial cells actively synthesized and secreted ECPg, just as in the PV epithelium. This fact has been repeatedly confirmed with the methods of chorioallantoic transplantation (Takiguchi *et al.*, 1986, 1988a), intracoelomic transplantation (Yasugi *et al.*, 1989a) and *in vitro* cultivation of Trowell type (Takiguchi *et al.*, 1988b). These results are important since they demonstrate almost the same ability to express ECPg in the epithelia of these organs under the favorite conditions provided by PV mesenchyme.

In contrast to the supportive influence of the PV mesenchyme, the GZ mesenchyme never permitted the expression of ECPg in the associated epithelia (Takiguchi *et al.*, 1986). Even the epithelium of PV, in which ECPg is expressed in normal development, could not express it when associated with GZ mesenchyme. Thus GZ mesenchyme has a potent inhibitory influence on the expression of ECPg.

Other mesenchymes of digestive organs such as the esophagus and small intestine showed intermediate effect. The epithelium of esophagus never synthesized ECPg on its own mesenchyme but small amounts of ECPg were observed in the esophageal epithelium associated with the small-intestinal mesenchyme, and the epithelia of PV and GZ also expressed small amount of ECPg on the mesenchymes of the esophagus and small intestine. Taken together, these results demonstrate that there are qualitative differences in the ability to regulate the expression of ECPg in the associated epithelia among the mesenchymes of these organs.

Reactivity of epithelia

The developmental fate of epithelial tissue is often determined by the associated mesenchyme, at least as regards the expression of ECPg. There is evidence, however, that the reactivity of epithelia is also important in some cases. Thus, the epithelia of the small intestine and allantois could not express ECPg when they were recombined and cultured with PV mesenchyme, although the epithelia formed complex glands which are not characteristic of these epithelia (Yasugi, 1984; Yasugi *et al.*, 1985). In this case, the absence of mRNA of ECPg in these epithelia was substantiated by Northern hybridization (Hayashi *et al.*, 1988c; Fig. 2B), suggesting that the control of ECPg expression is done at the transcription level rather than the post-transcription level. This experiment also revealed the independence of morphogenesis and cytodifferentiation in the epithelia (Mizuno and Yasugi, 1990).

We examined further the reactivity of very young, presumptive intestinal endoderm to the influence of PV mesenchyme of 6-day embryo. The presumptive developmental fate of young endoderm

of 1.5-2.0 day embryos had been precisely determined by the experiments of Le Douarin (1964). We took presumptive stomach (PV and GZ) and intestinal endodermal fragments and recombined them with PV mesenchyme and cultivated on the chorioallantoic membrane. Both endoderms formed glandular structures but only the presumptive stomach endoderm expressed ECPg mRNA and protein, detected by *in situ* hybridization and immunohistochemistry (Yasugi *et al.*, 1991). We are therefore tempted to conclude that the potentiality to express ECPg is lost early in the endoderm destined to develop into the intestinal and allantoic epithelia. This difference in the reactivity to PV mesenchyme may be a very early sign of regional differentiation in the endoderm.

The nature of mesenchymal influences

The most important and at the same time the most difficult problem in the study of tissue interaction is to find out the molecules responsible for the transmission of the mesenchymal influences. Despite the many works carried out so far, the exact nature of the mesenchymal influences is still obscure in many tissue interaction systems. We analyzed the biological characteristics of the mesenchymal influence, with special attention to the difference in PV and GZ mesenchymes.

Whether the mesenchymal influence can pass through the filters or not is an important question when the nature of the mesenchymal induction is considered. We inserted the Nuclepore filters of various pore sizes between PV mesenchyme and PV or GZ epithelium and cultivated the recombinants *in vitro*. After 6 days' culture, the epithelium formed gland-like structures and expressed ECPg when the pore size of the filter was larger than 0.6 μm . With the filters having a pore size larger than 0.6 μm , the penetration of the mesenchymal cells was observed on the epithelial surface of the filter (Takiguchi-Hayashi and Yasugi, 1990). So we concluded that, at least in the case of PV mesenchyme, the direct contact of mesenchymal cells and epithelial cells is necessary for the expression of ECPg in the epithelium, suggesting the importance of cell surface or extracellular substances of the mesenchymal cells in the process.

As for the GZ mesenchyme, the situation is somewhat different. We dissociated the mesenchymal cells of PV and GZ to single cells and mixed them in various ratios. The reaggregated mesenchymal cell mass was recombined with the GZ epithelium and cultivated. The dissociated and reaggregated mesenchymal cells supported the differentiation of the epithelium just as the intact mesenchyme. To our great surprise, the mixing of small amounts of GZ mesenchymal cells (10-25%) to PV mesenchymal cells resulted in a strong inhibitory action on the expression of ECPg in the epithelium. When the ratio of PV and GZ mesenchymal cells was 1:1, there occurred virtually no ECPg expression (and gland formation) in the epithelium. The similar inhibitory effect was observed with the esophageal mesenchymal cells, whereas the mesenchymal cells of the small intestine, lung and dorsal dermis showed no inhibitory effect (Urase and Yasugi, 1993). These experiments suggest that the GZ mesenchymal cells exert their inhibitory effect by some soluble, far-reaching factors, rather than by direct contact with the epithelial cells.

So far no information has been obtained with the molecular nature of the influences of PV or GZ mesenchymal cells, which modulate the expression of ECPg in the epithelium. We tried several times to prepare the monoclonal antibodies that recognize the difference between PV and GZ mesenchyme. A monoclonal antibody, T95, stained differently these two mesenchymes when

applied to the histological sections of PV and GZ of 6-day embryos. T95 antigen was detected amply in cells of the mesenchyme of GZ just under the epithelium, but was detected only faintly at the epithelial-mesenchymal interface in PV (Takiguchi-Hayashi and Yasugi, 1991). Although the precise nature of the T95 antigen was not clear, it revealed for the first time the existence of a molecular difference in these two mesenchymes.

Keratin expression and ECPg expression

Almost all epithelial cells express keratins (cytokeratins) as intermediate filaments. We were interested in the function of keratin in the differentiation of digestive organs and studied its expression in the epithelium during development. We used the monoclonal antibody PKK1, which was prepared against a swine kidney epithelial cell line and recognizes keratin 8, 18 and 19 in the catalogue of human keratins. PKK1 stained intracellular filamentous structures in cells of the digestive tract epithelium facing the lumen, whereas gland cells expressing ECPg become negative to these antibodies as development proceeds. PKK1-reactivity vanished almost completely in the latter half of embryonic development in the PV gland cells. This phenomenon elicited the question of great interest: whether the formation of glands, expression of ECPg and the disappearance of keratin recognized by PKK1 (PKK1 keratin) are controlled by the same action of PV mesenchyme?

We then conducted the experiment in which the expression of ECPg and PKK1 keratin was examined after the recombination of various epithelia and mesenchymes. The results were very clear; whenever cells came to express ECPg under the influence of PV mesenchyme, these cells lost their reactivity to PKK1. In contrast, cells not expressing ECPg remained reactive to PKK1. The epithelia of small intestine and allantois, which formed well-developed glandular structures under the influence of PV mesenchyme, but did not express ECPg, were positive to PKK1 (Takiguchi-Hayashi *et al.*, submitted). We therefore postulated that the expression of ECPg and the disappearance of PKK1 antigen are in close relation, possibly regulated by the same action of the mesenchyme.

If this is indeed the case, how do the mesenchymal factors bring about the activation of ECPg gene and inactivation of PKK1 antigen genes, at the same time? We can assume several possible scenarios: (1) the mesenchymal factors first inactivate PKK1 antigen gene and this triggers the activation of ECPg gene. (2) Inversely it is ECPg gene that is activated first by the factors and activated ECPg gene in turn inactivates PKK1 antigen gene. (3) The mesenchymal factors affect independently these two genes. (4) The mesenchymal factors activate first a key gene of which transcripts or products activate ECPg gene and inactivate PKK1 antigen gene.

Although we have at present no basis for judging which possibility is the most plausible one, we could exclude the second possibility because the disappearance of PKK1 antigen occurs later than the appearance of ECPg. I consider the last possibility most plausible, because it explains well the timing of the appearance and disappearance of two gene products. We can designate this key gene as a «master» gene of PV gland epithelial cells since the activity of this gene determines the developmental fate of cells in which this gene is transcribed, to be gland epithelial cells. To test this hypothesis, it is necessary first to clone PKK1 antigen gene and compare the modes of regulation of ECPg gene and PKK1 antigen gene. Relevant to this, it was recently shown that the many genes coding exocrine and endocrine proteins (hormones and enzymes) of the pancreas have common sequences to which the same

transcription factor can bind (Kruse *et al.*, 1993). If a gene coding this transcription factor is activated in undifferentiated endodermal cell populations, they would be determined as pancreatic epithelial cells. This gene can thus be called the master gene of pancreatic epithelial cells.

Regulation of ECPg gene expression through the regulatory region

Whatever the mechanisms of the mesenchymal actions are, the final regulation of ECPg gene must be done at the transcriptional level. Since the regulation of many genes so far known involves their activation or inhibition by the regulatory region located at 5' upstream region of the genes, we searched the region responsible for the regulation of ECPg gene by the mesenchymal influence.

For this purpose, we constructed plasmids consisting of 5' region of ECPg gene and the luciferase gene as a reporter gene. The construct was introduced into epithelial cells of PV or GZ of 6-day embryos by lipofection. Next, epithelial cells were mixed with mesenchymal cells of PV or GZ, and mixed cell aggregates were cultivated *in vitro*. After cultivation, the expression of luciferase gene was assayed by the photochemical method.

The luciferase activity in the aggregates composed of PV or GZ epithelial cells and PV mesenchymal cells was much higher than that of aggregates composed of epithelial cells and GZ mesenchymal cells (Fig. 3). By these methods, we were able to demonstrate that the regulation of ECPg gene expression by the mesenchyme is mediated via 5' regulatory region of the gene (Fukuda *et al.*, unpublished results).

The organ- and developmental stage-specific expression of many genes has been shown to be controlled by so-called enhancer elements which locate either in the upstream or downstream or in the middle of the genes. In some cases the mode of action of enhancer elements has been well analyzed (for instance, in the case of globin genes. See Engel, 1993). However, the enhancer elements mediating influence of mesenchyme to a definite gene have not been made known, as far as the present author is aware. The identification of the element will open a new way to analyze the molecular mechanisms involved in the regulation of ECPg gene by the mesenchyme.

Hypothesis and perspectives

I described here the present status of the study on the regulation of ECPg gene, which is a marker gene of PV epithelial cells, with much stress on the regulation by tissue interactions. We have elucidated the importance of mesenchymal influence, reactivity of epithelial tissue and enhancer element of the gene, and analyzed the molecular aspects of each step. We can now depict the rough scenario of the events occurring during the regulation of ECPg gene expression by the mesenchyme. The epithelia of the esophagus, PV and GZ are endowed with the innate ability to express ECPg under appropriate conditions, which, in normal development, are provided by PV mesenchyme probably with direct contact with epithelial cells. On the other hand, the mesenchymes of the esophagus and GZ exert an inhibitory effect on the ECPg expression in the epithelia by far-reaching, possibly soluble factors. With these actions of the mesenchymes of the esophagus, PV and GZ, only the epithelium of PV comes to express ECPg gene in the normal course of development, whereas the epithelia of the esophagus and GZ become inactive as to the expression of ECPg. The epithelium of the intestine has no

potential for ECPg expression from the early stage of development and hence the intestinal mesenchyme has no inhibitory action. The regional difference in the ability to express ECPg gene is further stabilized by the hypomethylation of ECPg gene in PV epithelial cells.

In epithelial cells, the expression of ECPg gene is controlled by the upstream sequence which is important both for transcription and for determination of organ-specific expression of the gene. The difference in the potency to express ECPg gene among various epithelia may be due to the existence or absence of some transcription factors in cells.

The scenario mentioned above can explain the organ-specific expression of ECPg gene in the development of the chicken embryo, without any contradiction to the experimental evidence so far obtained. There remain, however, many problems to be solved. First, the molecular nature of the mesenchymal action is still obscure. We are trying to extract factors responsible for the transmission of the mesenchymal influence to the epithelium by microanalytical methods and monoclonal antibody method. The most difficult problem in this study is to assay the effect of candidate substances on the expression of ECPg *in vitro*, since so far we have no epithelial cell line which can express it without the support of PV mesenchyme. To overcome this difficulty we are trying to establish a permanent line of PV epithelial cells by introducing genes of large T antigen of SV40 virus (Tabata *et al.*, unpublished result).

The intracellular signaling of information from the mesenchyme in epithelial cells is also an important aspect to be studied. The information coming from the mesenchyme must be picked up by epithelial cells by some receptors and be transmitted to nuclei to exert its final effect. We can suppose the involvement of the signal transduction system including the phosphorylation processes but the detailed analysis has not been done.

The nuclear factors regulating the transcription of ECPg will be elucidated in the near future because we have already determined the sequence to which nuclear factors bind. However, whether the expression of these nuclear factors in epithelial cells is regulated with the direct or indirect mesenchymal influences must be clarified.

Last but not least, we must always bear in mind the phylogenetic significance of ECPg expression in considering the mechanisms of its regulation. Pepsinogen genes originated early in the history of life and have evolved according to the adaptive life of each animal group. This fact must be affected in the mode of regulation of pepsinogen genes. Pepsinogen genes, especially ECPg gene which is expressed strictly organ- and developmental stage-specifically, will give us a very fascinating system to study the evolution and regulation of genes.

Acknowledgments

I wish to express my thanks to Prof. T. Mizuno for his continuous guidance in my work. My work described in this article was supported in part by grants from the Ministry of Education, Science and Culture of Japan and Tokyo Metropolitan University.

References

- CEDAR, H. and RAZIN, A. (1990). DNA methylation and development. *Biochim. Biophys. Acta* 1049: 1-8.
- ENGEL, J.D. (1993). Developmental regulation of human β -globin gene transcription. *Trends Genet.* 9: 304-309.
- EVERS, M.P.J., ZELLE, B., BEBELMAN, J.P., PRONK, J.C., MAGER, W.H., PLANTA, R.J., ERIKSSON, A.W. and FRANTS, R.R. (1988). Cloning and sequencing of

- Rhesus monkey pepsinogen A cDNA. *Gene* 65: 179-185.
- FUKUDA, K., ICHINOSE, M., SAIGA, H., SHIOKAWA, K. and YASUGI, S. (1994). Developmental changes of DNA methylation pattern of embryonic chick pepsinogen gene. *J. Biochem.* 115: 502-506.
- FURIHATA, C., KAWACHI, T. and SUGIMURA, T. (1972). Premature induction of pepsinogen in developing rat gastric mucosa by hormones. *Biochem. Biophys. Res. Commun.* 47: 705-711.
- HAYANO, T., SOGAWA, K., ICHIHARA, Y., FUJII-KURIYAMA, Y. and TAKAHASHI, K. (1988). Primary structure of human pepsinogen C gene. *J. Biol. Chem.* 263: 1382-1385.
- HAYASHI, K., AGATA, K., MOCHII, M., YASUGI, S., EGUCHI, G. and MIZUNO, T. (1988a). Molecular cloning and the nucleotide sequence of cDNA for embryonic chicken pepsinogen: phylogenetic relationship with chymosin. *J. Biochem.* 103: 290-296.
- HAYASHI, K., YASUGI, S. and MIZUNO, T. (1988b). Isolation and structural analysis of embryonic chicken pepsinogen gene: avian homologue of prochymosin gene. *Biochem. Biophys. Res. Commun.* 152: 776-782.
- HAYASHI, K., YASUGI, S. and MIZUNO, T. (1988c). Pepsinogen gene transcription induced in heterologous epithelial-mesenchymal recombinations of chicken endoderms and glandular stomach mesenchyme. *Development* 103: 725-731.
- HIDAKA, M., SASAKI, K., UOZUMI, T. and BEPPU, T. (1986). Cloning and structural analysis of the calf prochymosin gene. *Gene* 43: 197-203.
- HIRSCH-MARIE, H., LOISILLIER, F., TOUBOUL, J.P. and BURTIN, P. (1976). Immunohistochemical study and cellular localization of human pepsinogens during ontogenesis and in gastric cancers. *Lab. Invest.* 34: 623-632.
- ICHINOSE, M., MIKI, K., FURIHATA, C., TATEMATSU, M., ICHIHARA, Y., ISHIHARA, T., KATURA, I., SOGAWA, K., FUJII-KURIYAMA, Y., TANJI, M., OKA, H., MATSUSHIMA, T. and TAKAHASHI, K. (1988a). DNA methylation and expression of the rat pepsinogen gene in embryonic, adult and neoplastic tissues. *Cancer Res.* 48: 1603-1609.
- ICHINOSE, M., MIKI, K., TATEMATSU, M., FURIHATA, C., NOBUHARA, M., ICHIHARA, Y., TANJI, M., SOGAWA, K., FUJII-KURIYAMA, Y., OKA, H., TAKAHASHI, T., KAGEYAMA, T. and TAKAHASHI, K. (1988b). Hypomethylation and expression of pepsinogen A genes in the fundic mucosa of human stomach. *Biochem. Biophys. Res. Commun.* 151: 275-282.
- ICHINOSE, M., MIKI, K., TATEMATSU, M., MIZUNO, T., MUTAI, M., FURIHATA, C., ICHIHARA, Y., ISHIHARA, T., TANJI, M., OKA, H., HINOHARA, Y., TAKAHASHI, T., KAGEYAMA, T. and TAKAHASHI, K. (1988c). Cell-specific hypomethylation of the pepsinogen gene in pepsinogen-producing cells. *Biochem. Biophys. Res. Commun.* 155: 670-677.
- ISHIHARA, T., ICHIHARA, Y., HAYANO, T., KATSURA, I., SOGAWA, K., FUJII-KURIYAMA, Y. and TAKAHASHI, K. (1989). Primary structure and transcriptional regulation of rat pepsinogen C gene. *J. Biol. Chem.* 264: 10193-10199.
- KAGEYAMA, T., TANABE, K. and KOIWA, O. (1990). Structure and development of rabbit pepsinogens. Stage-specific zymogens, nucleotide sequences of cDNAs, molecular evolution, and gene expression during development. *J. Biol. Chem.* 265: 17031-17038.
- KRUSE, F., ROSE, S.D. and SWIFT, G.H. (1993). An endocrine-specific element is an integral component of an exocrine-specific pancreatic enhancer. *Genes Dev.* 7: 774-786.
- LE DOUARIN, N. (1964). Etude expérimentale de l'organogenèse du tube digestif et du foie chez l'embryon de Poulet. *Bull. Biol. Fr. Belg.* 98: 543-676.
- MIZUNO, T. and YASUGI, S. (1990). Susceptibility of epithelia to directive influences of mesenchymes during organogenesis: uncoupling of morphogenesis and cytodifferentiation. *Cell Differ. Dev.* 31: 151-159.
- MOIR, D., MAO, J.-I., SCHUMM, J.W., VOVIS, G.F., ALFORD, B.L. and TAUNTON-RIGBY, A. (1982). Molecular cloning and characterization of double-stranded complementary DNA coding for bovine chymosin. *Gene* 19: 127-138.
- MUTO, N. and TANI, S. (1979). Purification and characterization of rat pepsinogens whose contents increase with developmental progress. *J. Biochem.* 85: 1143-1149.
- NORTHROP, J.P. (1930). Crystalline pepsin. I. Isolation and tests of purity. *J. Gen. Physiol.* 13: 739-766.
- SOGAWA, K., FUJII-KURIYAMA, Y., MIZUKAMI, Y., ICHIHARA, Y. and TAKAHASHI, K. (1983). Primary structure of human pepsinogen gene. *J. Biol. Chem.* 258: 5306-5311.
- SOGAWA, K., ICHIHARA, Y., TAKAHASHI, K., FUJII-KURIYAMA, Y. and MURAMATSU, M. (1981). Molecular cloning of complementary DNA to swine pepsinogen mRNA. *J. Biol. Chem.* 256: 12561-12565.
- TAGGERT, R.T., CASS, L.G., MOHANDAS, T.K., DERBY, P., BARR, P.J., PALS, G. and BELL, G.I. (1989). Human pepsinogen C (progastricscin). Isolation of cDNA clones, localization to chromosome 6, and sequence homology with pepsinogen A. *J. Biol. Chem.* 264: 375-379.
- TAKIGUCHI, K., YASUGI, S. and MIZUNO, T. (1986). Gizzard epithelium of chick embryos can express embryonic pepsinogen-antigen, a marker protein of proventriculus. *Roux Arch. Dev. Biol.* 195: 475-483.
- TAKIGUCHI, K., YASUGI, S. and MIZUNO, T. (1988a). Developmental changes in the ability to express embryonic pepsinogen in the stomach epithelia of chick embryos. *Roux Arch. Dev. Biol.* 197: 56-62.
- TAKIGUCHI, K., YASUGI, S. and MIZUNO, T. (1988b). Pepsinogen induction in chick stomach epithelia by reaggregated proventricular mesenchymal cells *in vitro*. *Dev. Growth Differ.* 30: 241-250.
- TAKIGUCHI-HAYASHI, K. and YASUGI, S. (1990). Transfilter analysis of the inductive influence of proventricular mesenchyme on stomach epithelial differentiation of chick embryos. *Roux Arch. Dev. Biol.* 198: 460-466.
- TAKIGUCHI-HAYASHI, K. and YASUGI, S. (1991). Localized distribution of a novel mesenchyme-specific antigen in developing chick digestive organs. Comparison with the distribution of fibronectin, laminin and tenascin. *Roux Arch. Dev. Biol.* 200: 113-116.
- TONER, P.G. (1963). The fine structure of resting and active cells in the submucosal glands of the fowl proventriculus. *J. Anat.* 97: 575-583.
- TROWELL, O.A. (1954). A modified technique for organ culture *in vitro*. *Exp. Cell Res.* 6: 246-258.
- URASE, K. and YASUGI, S. (1993). The induction and inhibition of epithelial differentiation by the mixed cell aggregates of the mesenchymes from the chicken embryonic digestive tract. *Dev. Growth Differ.* 35: 33-40.
- VONK, H.J. and WESTERN, J.R.H. (1984). *Comparative Biochemistry and Physiology of Enzyme Digestion*. Academic Press, New York.
- WOLFF, Et. and HAFFEN, K. (1952). Sur une méthode de culture d'organes embryonnaires *in vitro*. *Texas Rep. Biol. Med.* 10: 463-472.
- YAKABE, E., TANJI, M., ICHINOSE, M., GOTO, S., MIKI, K., KUOKAWA, K., ITO, H., KAGEYAMA, T. and TAKAHASHI, K. (1991). Purification, characterization and amino acid sequences of pepsinogens and pepsins from the esophageal mucosa of bullfrog (*Rana catesbeiana*). *J. Biol. Chem.* 266: 22436-22443.
- YASUGI, S. (1984). Differentiation of allantoic endoderm implanted into the presumptive digestive area in avian embryos. A study with organ-specific antigens. *J. Embryol. Exp. Morphol.* 80: 137-153.
- YASUGI, S. (1987). Pepsinogen-like immunoreactivity among vertebrates: occurrence of common antigenicity to an anti-chicken pepsinogen antiserum in stomach gland cells of vertebrates. *Comp. Biochem. Physiol.* 86B: 675-680.
- YASUGI, S. (1993). Role of epithelial-mesenchymal interactions in differentiation of epithelium of vertebrate digestive organs. *Dev. Growth Differ.* 35: 1-9.
- YASUGI, S. and MIZUNO, T. (1981a). Developmental changes in acid proteases of the avian proventriculus. *J. Exp. Zool.* 216: 331-335.
- YASUGI, S. and MIZUNO, T. (1981b). Purification and characterization of embryonic chicken pepsinogen, a unique pepsinogen with large molecular weight. *J. Biochem.* 89: 311-315.
- YASUGI, S. and MIZUNO, T. (1990). Mesenchymal-epithelial interactions in the organogenesis of digestive tract. *Zool. Sci.* 7: 159-170.
- YASUGI, S., HAYASHI, K., TAKIGUCHI, K., MIZUNO, T., MOCHII, M., KODAMA, R., AGATA, K. and EGUCHI, G. (1987). Immunological relationships among embryonic and adult chicken pepsinogens: a study with monoclonal and polyclonal antibodies. *Dev. Growth Differ.* 29: 85-91.
- YASUGI, S., KEDINGER, M., SIMON-ASSMANN, P., BOUZIGES, F. and HAFFEN, K. (1989a). Differentiation of proventricular epithelium in xenoplastic associations with mesenchymal or fibroblastic cells. *Roux Arch. Dev. Biol.* 198: 114-117.
- YASUGI, S., MATSUMOTO, F. and MIZUNO, T. (1987). Evolution des pepsinogènes au cours du développement, chez la Souris. *C. R. Soc. Biol. (Paris)* 181: 450-453.
- YASUGI, S., MATSUNAGA, T. and MIZUNO, T. (1988). Presence of pepsinogens immunoreactive to anti-embryonic chicken pepsinogen antiserum in fish stomachs: possible ancestor molecules of chymosin of higher vertebrates. *Comp. Biochem. Physiol.* 91A: 565-569.
- YASUGI, S., MATSUNAGA, T. and MIZUNO, T. (1989b). Pepsinogen-like immunoreactivity in ascidian stomach and intestine: immunohistochemical and biochemical study. *Zool. Sci.* 6: 283-288.
- YASUGI, S., MATSUSHITA, S. and MIZUNO, T. (1985). Gland formation induced in the allantoic and small-intestinal endoderm by the proventricular mesenchyme is not coupled with pepsinogen expression. *Differentiation* 30: 47-52.
- YASUGI, S., TAKEDA, H. and FUKUDA, K. (1991). Early determination of developmental fate in presumptive intestinal endoderm of the chicken embryo. *Dev. Growth Differ.* 33: 235-241.