Pepsinogen gene transcription induced in heterologous epithelial–mesenchymal recombinations of chicken endoderms and glandular stomach mesenchyme

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Summary

Proventricular (glandular stomach) mesenchyme of chicken embryos can induce endoderms of some parts of embryonic digestive tract to produce embryonic chicken pepsinogen (ECPg), a marker protein for the differentiation of embryonic proventricular epithelium. In the present study, we investigated the production of ECPg mRNA in the course of epithelial–mesenchymal interactions between endoderms of digestive tract and proventricular mesenchyme. ECPg mRNA was detected by Northern hybridization with ECPg cDNA as a probe. In normal development of the proventriculus, ECPg mRNA was first detected at day 7 of incubation, and it ceased to be produced by day 21. Embryonic esophagus, gizzard and small intestine did not contain ECPg mRNA. When 6-day esophagus, gizzard or proventricular endoderm was associated

and cultured with 6-day proventricular mesenchyme, the recombinates formed proventricular-gland-like complex glands and produced ECPg mRNA in almost equal quantity. However, 6-day small intestinal or 3·5-day allantoic endoderm did not produce pepsinogen mRNA under the same conditions, though the recombinates formed complex glands. These results indicate that the proventricular mesenchyme can induce de novo transcription of ECPg gene in esophagus, proventricular and gizzard endoderms, and that ECPg gene in small intestinal and allantoic endoderms fails to react to the inducing signal.

Key words: embryonic chicken pepsinogen, digestive tract, epithelial–mesenchymal interaction, gene expression, transcription, Northern hybridization.

Introduction

Epithelial–mesenchymal interactions play an important role in various kinds of organogenesis of vertebrate embryos (reviewed by Wessells, 1977; Kratochwil, 1982; Sawyer & Fallon, 1983). The interactions between the two tissues often induce morphogenesis and differentiation of one or both tissues. For instance, morphogenesis and differentiation of avian proventricular epithelium are induced by the surrounding mesenchyme. The inducing action of proventricular mesenchyme has been demonstrated in heterologous recombinations. If endoderms of various parts of embryos, such as esophagus, gizzard, small intestine, allantois and yolk sac,

are recombined and cultured with proventricular mesenchyme, morphogenesis characteristic of proventricular epithelium is induced (Sigot, 1971; Yasugi & Mizuno, 1974, 1978; Gumpel-Pinot et al. 1978; Masui, 1981). In some cases, specific cytodifferentiation is also demonstrated to be induced. If gizzard endoderm or hypoblast is recombined with proventricular mesenchyme, the endoderm produces embryonic chicken pepsinogen (ECPg), a marker protein for cytodifferentiation of the proventricular epithelium (Yasugi & Mizuno, 1984; Takiguchi et al. 1986). These investigations suggest that proventricular mesenchyme influences the pattern of gene expressions in the endoderm, although the molecular mechanisms working in the process are not yet well

understood.

The molecular mechanisms involved in cell-type specific production have been studied intensively. It is known that cell-type-specific production is regulated not only at the level of gene transcription, but also at the levels of mRNA processing, modulation of mRNA stability, translation of the mRNA and protein processing. It is interesting to investigate at which level the production of ECPg is induced in endoderms by the proventricular mesenchyme.

In contrast to the cases in which both heterotypic morphogenesis and cytodifferentiation are induced, there are several cases in which heterotypic cytodifferentiation is not induced in spite of heterotypic morphogenesis (Sakakura *et al.* 1976; Lawson, 1972; Tyler & Koch, 1977; Yasugi, 1984; Yasugi *et al.* 1985). Yasugi (1984) showed that allantoic endoderm implanted into the presumptive stomach area of chicken embryos fails to synthesize ECPg, even if it is incorporated into the host proventriculus and forms proventricular glands. Endoderm of the small intestine is equally unable to synthesize ECPg under the influence of proventricular mesenchyme, despite its proventricular-gland-like morphogenesis (Yasugi *et al.* 1985).

Although these findings suggest that small intestinal and allantoic endoderms cannot react to the pepsinogen-inducing influence of proventricular mesenchyme, we must examine the possibility that ECPg gene is transcribed in these endodermal cells but the mRNA cannot be translated. This possibility is not inconceivable, because Agata & Eguchi (1984) showed that a precursor of δ-crystallin mRNA can be detected in the dedifferentiated chicken pigmented epithelial cells, but it is not translated. This RNA is processed and translated when the cells 'transdifferentiate' into lens cells under appropriate culture conditions (Itoh & Eguchi, 1986).

The purpose of the present study is to examine (1) at what level the expression of ECPg is controlled in the course of epithelial–mesenchymal interactions, and (2) whether or not the small intestinal or allantoic endoderm can express any ECPg mRNA under the influence of the proventricular mesenchyme. The availability of a cDNA clone for ECPg (Hayashi *et al.* 1988*a*) has allowed us to address directly these questions.

Materials and methods

Tissue recombination and grafting

Tissue recombination and grafting methods were the same as described by Yasugi *et al.* (1985). Esophagus, proventriculus, gizzard and small intestine were isolated from 6-day chicken embryos and allantois from 3·5-day embryos. The endoderms were separated from the mesenchyme by
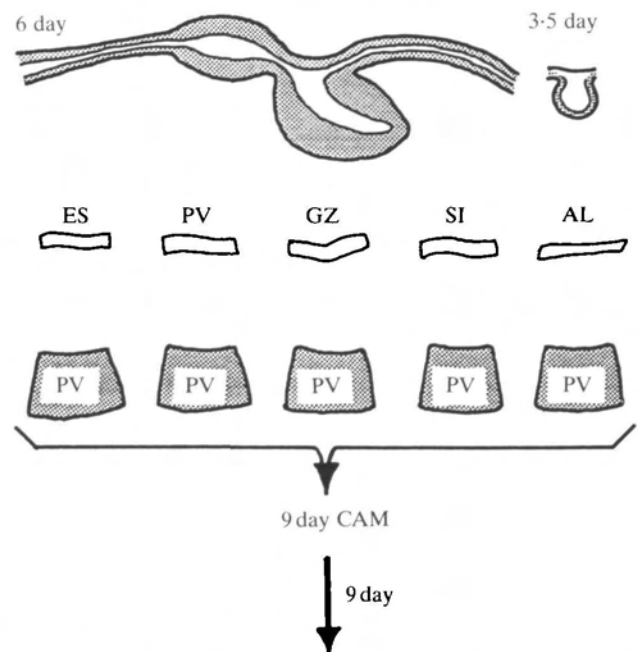

Fig. 1. Scheme of recombination experiments. Four parts of digestive endoderm of 6-day chicken embryos and allantoic endoderm of 3·5-day embryos were isolated, and associated with the proventricular mesenchyme of 6-day embryos. The recombinates were cultured for 9 days on the chorioallantoic membrane (CAM) of 9-day embryos. ES, esophagus; PV, proventriculus; GZ, gizzard; SI, small intestine; AL, allantois.
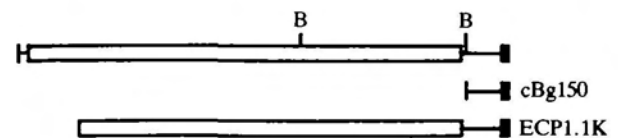

Fig. 2. cDNA probes used for Northern blot analysis. The full-length cDNA for ECPg is shown at the top. Open bars indicate the protein-coding region for ECPg. Solid boxes indicate poly(A) tails. ECP1.1K (1110 bp and poly(A)) contains 87 % nucleotides of full-length cDNA. cBg150 (110 bp and poly (A)) contains 3′ untranslated region and was used as a ECPg mRNA-specific probe. B, *Bgl*II cleavage sites.

collagenase digestion. These endoderms were associated with proventricular mesenchyme of 6-day embryos on an agar medium containing horse serum (Wolff & Haffen, 1952). After incubation of the recombinates on the agar medium overnight, they were grafted onto the chorioallantoic membrane of 9-day chicken embryos and cultured for 9 days at 37 °C (Fig. 1).

Probes

ECPg cDNA was cloned and the nucleotide sequence was already determined (Hayashi *et al.* 1988*a*). The full-length cDNA contains 1281 base pairs. There is a *Bgl*II cleavage site just at the end of the protein-coding region (Fig. 2).

The probes used for Northern blot analysis in this study were a *Bgl*II fragment (cBg150) that contains 110 bp of the 3' untranslated region and the poly(A) tail, and a 1·1 kb cDNA clone (ECP1.1K, refer to Hayashi *et al.* 1988*a*), which contains about 87 % of the full-length ECPg cDNA.

mRNA extraction and Northern blot analysis

Samples were homogenized with a Polytron homogenizer and total nucleic acids were extracted with phenol–chloroform. Poly(A)$^+$ RNA was isolated by oligo(dT)-cellulose chromatography according to Aviv & Leder (1972). RNA preparations were denatured in glyoxal, subjected to electrophoresis in 1 % agarose gels and blotted on a nylon membrane (Pall) in 20× SSC (1× SSC contains 0·15 M-NaCl and 0·015 M-sodium citrate). Radiolabelling of the probes was performed by nick translation (Nick Translation Kit, Amersham) with α-[^{32}P]dCTP. When cBg150 was used as a probe, the blots were hybridized in 6× SSC, 6× Denhardt's solution (1× Denhardt's solution contains 1 % bovine serum albumin, 1 % polyvinylpyrrolidone and 1 % Ficoll), 0·1 % SDS (sodium dodecyl sulphate), 100 μg ml^{-1} denatured salmon sperm DNA at 68°C for 18 h, and were washed twice in 2× SSC, 0·1 % SDS at 37°C for 15 min, and twice in 0·1× SSC, 0·1 % SDS at 37°C for 30 min. When ECP1.1K was used as a probe, the blots were hybridized in the same hybridizing solution at 50°C for 18 h, and were washed twice in 2× SSC, 0·1 % SDS at 37°C for 15 min. Rehybridization was performed after washing the membrane in 5 mM-Tris–HCl (pH 8·0), 0·2 mM-EDTA, 0·05 % sodium pyrophosphate, and 0·002× Denhardt's solution at 65°C for longer than 2 h. Hybrids were detected by autoradiography with an intensifying screen at −80°C.

Histological techniques

Samples were fixed in ice-cold 95 % ethanol for 4 h and embedded in paraffin. 5 μm sections were examined by indirect immunofluorescence or by staining with PAS–haematoxylin. Indirect immunofluorescence was performed according to Sainte-Marie (1962) with monoclonal antibody against ECPg (Y37; Yasugi *et al.* 1987) and the anti-mouse IgG antiserum conjugated with fluorescein isothiocyanate (Miles). The fluorescence was observed with an Olympus epifluorescent microscope.

Results

ECPg mRNA in the normal development of the digestive tract

First we investigated the time course of ECPg mRNA production during normal development of the proventriculus. Poly(A)$^+$ RNAs extracted from chicken proventriculi of various stages were electrophoresed in an agarose gel and blotted on a nylon membrane. The membrane was hybridized to cBg150 (see Fig. 2). Autoradiograms showed that ECPg mRNA was first detectable at day 7 (Fig. 3A), a day after the proventricular glands appear. The most abundant transcript was observed at day 15 (Fig. 3B; Note that panel A was exposed five times longer than panel B). The quantity of mRNA decreased rapidly after day 18. Adult proventriculus possessed no detectable ECPg mRNA. This time course of ECPg mRNA production matched well with the change of peptic activity in chicken embryonic proventriculus, which was shown to be highest at day 15 and decreased at day 20 (Yasugi & Mizuno, 1981), suggesting that ECPg mRNA is translated and degraded within not so long a period. It was also shown that ECPg mRNA was 1·6 kb in size throughout the embryonic stages in which it existed.

The membrane was then rehybridized to ECP1.1K, which contains a large part of the protein-coding region (see Fig. 2). A sharp cross-hybrid was observed at 1·6 kb in the lane of adult proventriculus (Fig. 3C). This may be mRNA for adult chicken pepsinogen, which has a considerable homology in
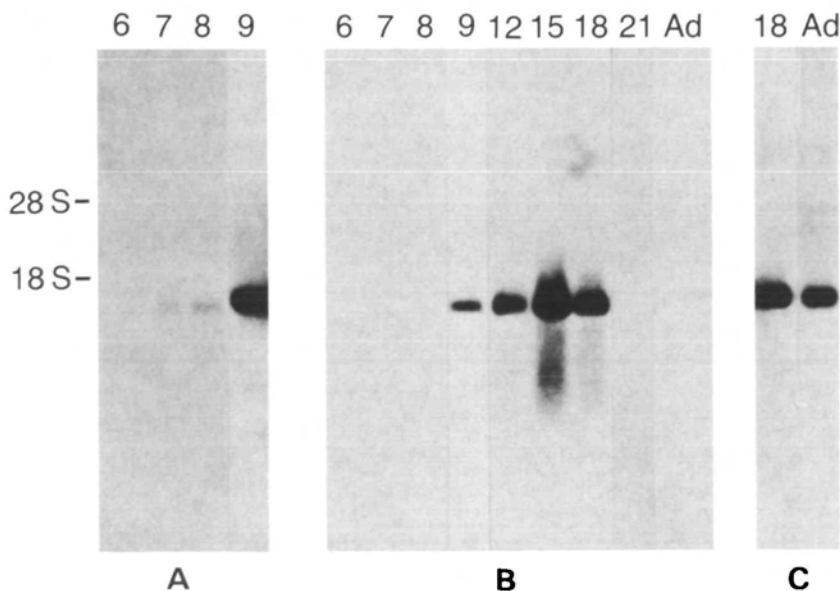

Fig. 3. The time course of ECPg mRNA expression during the normal development of chicken proventriculus. The numerals at the top indicate the days of incubation. 1 μg of poly(A)$^+$ RNA extracted from proventriculi of embryos or adult chicken (lane Ad) were subjected to Northern blot analysis. Blots were hybridized first with cBg150 (A,B). In A the membrane was exposed longer (75 h) than in B (15 h exposure). The blots were then rehybridized to ECP1.1K (C). The positions of 18S and 28S rRNA were indicated.
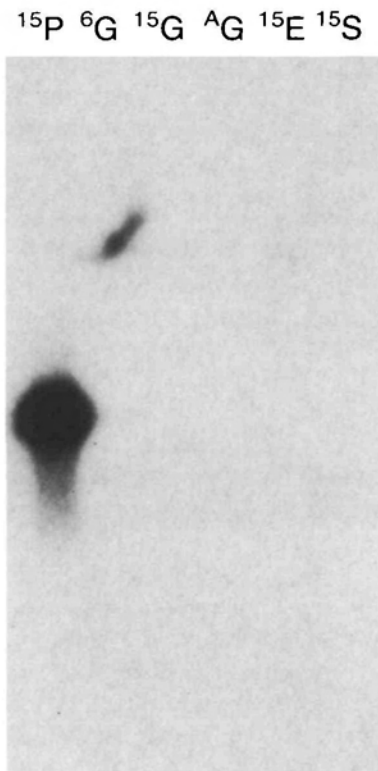
^{15}P ^{6}G ^{15}G ^{A}G ^{15}E ^{15}S

Fig. 4. Tissue specificity of ECPg mRNA expression in chicken digestive tract. $1\,\mu g$ of poly(A)$^{+}$ RNA extracted from 15-day proventriculus (^{15}P), 6-day gizzard (^{6}G), 15-day gizzard (^{15}G), adult gizzard (^{A}G), 15-day esophagus (^{15}E) and 15-day small intestine (^{15}S) were subjected to Northern blot analysis with cBg150 as a probe. The spot seen at the lane ^{15}G is an artifact.

amino acid sequence with ECPg (Hayashi *et al.* 1988*a*). This result indicates that the failure to detect ECPg mRNA in adult proventriculus with cBg150 was not due to degradation of the RNA preparation of adult proventriculi. The result also indicates that cBg150 hybridizes to ECPg mRNA specifically.

Second, ECPg mRNA expression in the other parts of the digestive tract of chicken embryos was investigated. Poly(A)$^{+}$ RNAs from 6-day, 15-day and adult gizzard, 15-day esophagus and 15-day small intestine were subjected to Northern blot analysis. These endoderms did not exhibit any hybrids with cBg150 (Fig. 4)

ECPg mRNA in endoderms associated and cultured with proventricular mesenchyme

Endoderms of esophagus, proventriculus, gizzard and small intestine of 6-day chicken embryos and allantoic endoderm of 3·5-day embryos were isolated and associated with proventricular mesenchyme of 6-day embryos. These recombinates were grafted on

the chorioallantoic membrane of 9-day chicken embryos and cultured for 9 days (Fig. 1).

In all kinds of recombinations, the endoderms formed compound glands characteristic of the proventriculus. Indirect immunofluorescence with the monoclonal antibody against ECPg revealed the presence of ECPg in the glandular cells developed from endoderms of esophagus, proventriculus and gizzard (Fig. 5A–C). On the contrary, endoderms of small intestine and allantois which formed complex glands under the influence of proventricular mesenchyme (Fig. 5D,F) did not synthesize any detectable ECPg (Fig. 5E,G).

We investigated the expression of ECPg mRNA in these recombinates. More than 25 recombinates of each combination were pooled and poly(A)$^{+}$ RNAs extracted from them were subjected to Northern blot analysis. When cBg150 was used as a probe, endoderms of esophagus, proventriculus and gizzard associated and cultured with proventricular mesenchyme exhibited distinct bands at 1·6 kb, which was the same size as the ECPg mRNA expressed in the normal proventriculus (Fig. 6). The intensity of these bands was measured with a densitometer and the amount of ECPg mRNA per μg of poly(A)$^{+}$ RNA transcribed in each of the three recombinates was estimated as about one sixth of the amount of that transcribed in 15-day proventriculus. In contrast, recombinates of intestinal and allantoic endoderms exhibited no RNA species that hybridized to cBg150. No signals appeared even when $10\,\mu g$ of poly(A)$^{+}$ RNA of each recombinate were applied (data not shown). To examine the possibility that pepsinogen mRNA other than that for ECPg was transcribed in these recombinates, we rehybridized the membrane with ECP1.1K, a probe that hybridized to adult pepsinogen mRNAs. No hybrids appeared on the lanes of recombinates of small intestinal or allantoic endoderm with proventricular mesenchyme (data not shown).

Discussion

Many studies have revealed that tissues can be induced to differentiate heterotypically and express new products in the course of epithelial–mesenchymal interactions (Sawyer & Fallon, 1983). However, it is still unclear whether the transcription of new genes is indeed induced. A report by Snead *et al.* (1984) suggested that *de novo* transcription of amelogenin gene occurs as a result of ectomesenchymal interaction in normal organogenesis of mouse molar tooth. However, they did not prove it by recombination experiments. As to the mesodermal induction in very early amphibian development, Gurdon *et al.*
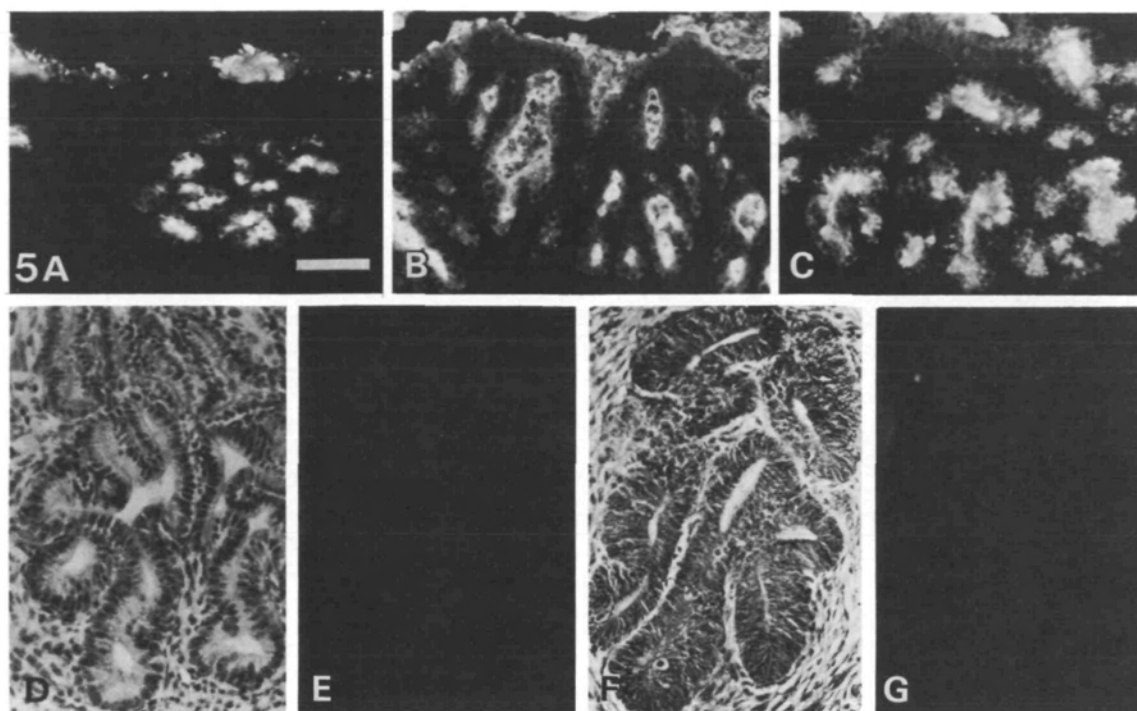
Fig. 5. Histology and immunofluorescence for ECPg of recombinates. Endoderms of esophagus (A), proventriculus (B), gizzard (C), small intestine (D,E) and allantois (F,G) were associated with the proventricular mesenchyme and cultured. D and F show the histology by PAS–haematoxylin staining. The others show the immunofluorescence with anti-ECPg monoclonal antibody. Bar, $50\,\mu m$.

(1985) demonstrated that transcription of cardiac actin gene is induced in the conjugates of animal and vegetal cells. In the present study, we demonstrated that proventricular mesenchyme can induce *de novo* transcription of ECPg mRNA in some heterologous endoderms.

Our data indicated that in normal development 6-day proventricular and gizzard endoderms do not produce detectable ECPg mRNA. The recombination experiments showed that 6-day endoderms of esophagus, proventriculus and gizzard produce ECPg mRNA in almost the same quantity (per μg poly(A)$^+$RNA) when combined and cultured with proventricular mesenchyme. The induction of ECPg synthesis in gizzard endoderm by proventricular mesenchyme was previously reported by Takiguchi *et al.* (1986, 1988), who detected ECPg by immunohistochemistry and enzymic assays. The present findings indicate the induction of *de novo* transcription of the ECPg gene by proventricular mesenchyme. However, the molecular process taking place during the transcriptional induction of ECPg gene is unknown. To elucidate this process, we have to study factors involved in the ECPg gene regulation. Recently we isolated an ECPg genomic clone and found two enhancer core consensus sequences within 400 bp upstream of the transcription start site (Hayashi *et al.* 1988*b*). It is possible that the sequences around them

may be responsible for proventriculus-specific expression or the induction of ECPg gene.

Endoderms of small intestine and allantois were not induced to produce ECPg mRNA. This result excludes untranslatable ECPg mRNA as a cause for the failure to produce the enzyme (See Introduction). Furthermore, no hybrids were detected in these recombinates even with ECP1.1K as a probe (data not shown), which cross-hybridized with adult-type pepsinogen mRNA (Fig. 3C). This suggests that these recombinates did not possess mRNA for any isoforms of pepsinogen, whereas they showed proventricular-gland-like morphogenesis. Thus, dissociation of morphogenesis and cytodifferentiation at the transcriptional level was demonstrated. Morphogenesis of proventricular gland was studied previously (Hayashi, 1987), and elevated mitotic activity at the tip of the elongating glands was observed. The glandular cell proliferation is supposed to be indispensable for proventricular gland elongation into the mesenchyme. Although the mechanisms involved in the glandular cell proliferation are unknown, the cell proliferation may not be coregulated with pepsinogen gene activation.

6-day endoderms of esophagus and gizzard could be induced to express ECPg mRNA in similar amounts to proventricular endoderm, indicating that the developmental fate of these endoderms on day 6
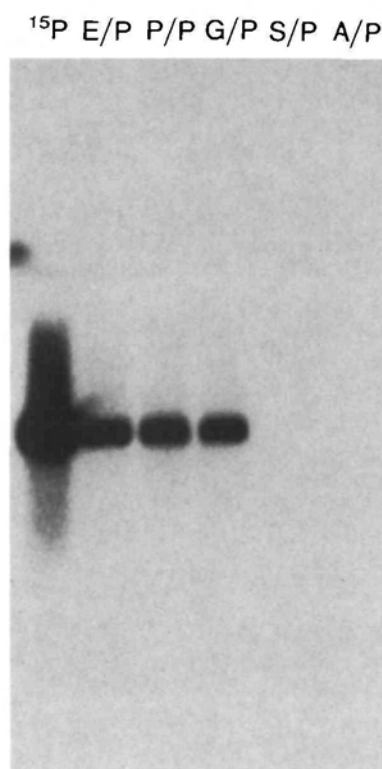
^{15}P E/P P/P G/P S/P A/P

Fig. 6. Northern blot analysis for ECPg mRNA of recombinates. Endoderms of the esophagus (E/P), proventricular (P/P), gizzard (G/P), small intestine (S/P) and allantois (A/P) were associated and cultured with the proventricular mesenchyme. 1 µg of poly(A)$^+$ RNA extracted from each recombinate was subjected to Northern blot analysis with cBg150 as a probe. 1 µg poly(A)$^+$ RNA of 15-day proventriculus was also loaded on far left lane (^{15}P) to indicate the normal size and quantity of ECPg mRNA.

were not yet determined as regards the ECPg expression. In contrast, the developmental fate of 6-day small intestinal endoderm seems to be already determined. It was previously shown that 6-day small intestinal endoderm has an intensive tendency to differentiate into small intestinal epithelium even under the influence of mesenchymes from other parts of the digestive tract (Yasugi & Mizuno, 1978). This determination might occur in very early stages of development. It has been shown by mesenchyme-free culture that some endodermal cells at stage 4 of Hamburger & Hamilton (1951) can selfdifferentiate into small intestinal epithelium (Sumiya & Mizuno, 1976; Mizuno & Sumiya, 1977; Ishizuya-Oka, 1983), although it has not yet been tested whether these young cells can differentiate into small intestinal cells under the influence of heterologous mesenchyme. As to the allantoic endoderm, Matsushita (1984) reported that 3·5-day allantoic endoderm differen-

tiates into the small intestinal and/or cloacal epithelium under the presence of any kind of digestive-tract mesenchyme.

Why did the endoderms of the small intestine and allantois fail to react to the ECPg-inducing action of the proventricular mesenchyme, while the endoderms of organs in the anterior part of the digestive tract (esophagus, proventriculus and gizzard) reacted? One possible answer is that transcription of the ECPg genes in the small intestinal and allantoic endodermal cells is blocked by methylation or undemethylation of DNA (Keshet *et al.* 1985), or by formation of inactive chromatin structure of ECPg gene (Wu & Gilbert, 1981). It may also be possible that some factors taking part in the process of the induction of ECPg expression, such as pre-existing *trans*-acting transcription factors (Ranjan & Baltimore, 1986) that activate ECPg gene after they are activated, or receptor molecules that receive inducing substances at the surface of the reacting cells (Takata *et al.* 1981), are missing in small intestinal and allantoic endodermal cells. The mechanisms responsible for the inability of the small intestinal and allantoic endoderms to be induced by the proventricular mesenchyme may be profoundly correlated with the nature of the determination of the developmental fate of these endoderms.

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