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Location of pre-hepatic cells in the early developmental stages of quail embryos

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SUMMARY

The location of the pre-hepatic cells which can respond to the inductive influence of the cardiac mesoderm and differentiate to the hepatic epithelium was investigated in quail embryos between the unincubated and 8-somite stages.

These cells were found to exist in the whole area of the blastoderm of unincubated and pre-streak stages. At the short-streak stage, just before the beginning of gastrulation, pre-hepatic cells are confined to the anterior part of the primitive streak. During gastrulation, pre-hepatic cells invaginate through this area of the primitive streak and enter the endoblastic layer. They are found in the anterior half of the endoblastic layer as well as in the lower half of the anterior part of the primitive streak at the medium- to definitive-streak stages. After gastrulation, they are consistently found only in the anterior half of the endoblastic layer. From the 1-somite stage, at least up to the 8-somite stage, the pre-hepatic cells are localized in the endoderm, anterior to the level of the 3rd somite.

Since the pre-hepatic cells or their progenitors were always found within the definitive endoblast which invaginates from the epiblast during gastrulation, it can be presumed that the pre-hepatic cells originate from the epiblast, invaginate through the anterior part of the primitive streak between the short- and the definitive-streak stage, and enter the definitive endoblast.

INTRODUCTION

The endoderm of chick and quail embryos acquires hepatogenic potency around the 2- to 5-somite stage by the inductive influence of the mesoderm of the precardiac and cardiac region (cardiac mesoderm) and differentiates into the hepatic epithelium at later developmental stages (Le Douarin, 1964*a*, *b*; Fukuda, 1979; Fukuda-Taira, 1981). The hepatic inductive potency can be demonstrated in the cardiac mesoderm *in vitro* as well as *in vivo* and is specific to the cardiac mesoderm (Fukuda, 1979; Fukuda-Taira, 1981). It has also been demonstrated that there is a specific endoderm which can respond to hepatic induction by the cardiac mesoderm. We called this endoderm pre-hepatic endoderm (Fukuda-Taira, 1981).

In the present investigation, the endoderm or the cells which can differentiate into the hepatic epithelium under the inductive influence of the cardiac mesoderm will be called *pre-hepatic endoderm* or *pre-hepatic cells.*

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The distribution of pre-hepatic cells has been studied by several investigators by chorioallantoic grafts (Hunt, 1931, 1932; Rudnick, 1932; Rawles, 1936), coelomic grafts, carbon marking method (Le Douarin, 1964*a*) and radioautographic mapping experiments (Rosenquist, 1971*a*). Mapping studies with chorioallantoic or coelomic grafts, however, failed to detect pre-hepatic cells when the hepatic inductor was absent from the explants. Carbon marking or radioautographic mapping studies demonstrated the precise area which enters the liver, but they did not necessarily show the distribution of cells which can differentiate into hepatic cells.

In the present study, distribution of pre-hepatic cells or endoderm in quail embryos between the unincubated and 8-somite stages was studied by examining the responsiveness of pieces of blastoderms to cardiac mesoderm.

MATERIALS AND METHODS

Embryos

Japanese quail (*Coturnix coturnix japonica*) and White Leghorn chicken (*Gallus gallus domesticus*) embryos were used.

Blastoderms were staged by the application of Vakaet (1970) or Eyal-Giladi & Kochav (1976) to quail embryos as follows or by the number of paired somites.

Unincubated stage (*stage X to XII of Eyal-Giladi & Kochav*). A freshly laid egg. Before hypoblast formation.
Pre-streak stage (*stage 1 of Vakaet*). The blastoderm is composed of epiblast and hypoblast.
Short-streak stage (*stage 3 of Vakaet*). The anterior end of the primitive streak is behind the centre of the area centralis of the area pellucida. The primitive groove has not yet appeared.
Medium-streak stage (*stage 4 of Vakaet*). The anterior end of the primitive streak is in the centre of the area centralis, and the primitive groove appears.
Long-streak stage (*stage 5 of Vakaet*). Anterior end of the primitive streak is in front of the centre of the area centralis.
Definitive-streak stage (*stage 6 of Vakaet*). Anterior extension of the primitive streak is at its maximum. Hensen's node is distinct.
Head-process and *head-fold stages* correspond to *stage 8* and *9* of *Vakaet* respectively.

Dissection of the epiblast

Epiblastic fragments were obtained from unincubated to definitive-streak-stage quail blastoderms. For the unincubated stage, whole blastoderm was used. From blastoderm at the pre-streak stage, whole epiblast was isolated mechanically from the hypoblast.

Blastoderms between the short-streak and the definitive-streak stage were cut at anteroposterior level into anterior and posterior halves. From each half, the epiblastic fragment was isolated by collagenase treatment (Worthington, CLS, 0·03 % in Tyrode's solution for 60 min at 37 °C). After separation, tissue fragments were washed thoroughly in three changes of serum-supplemented Tyrode's solution and finally in fresh Tyrode's solution. The epiblastic fragments include

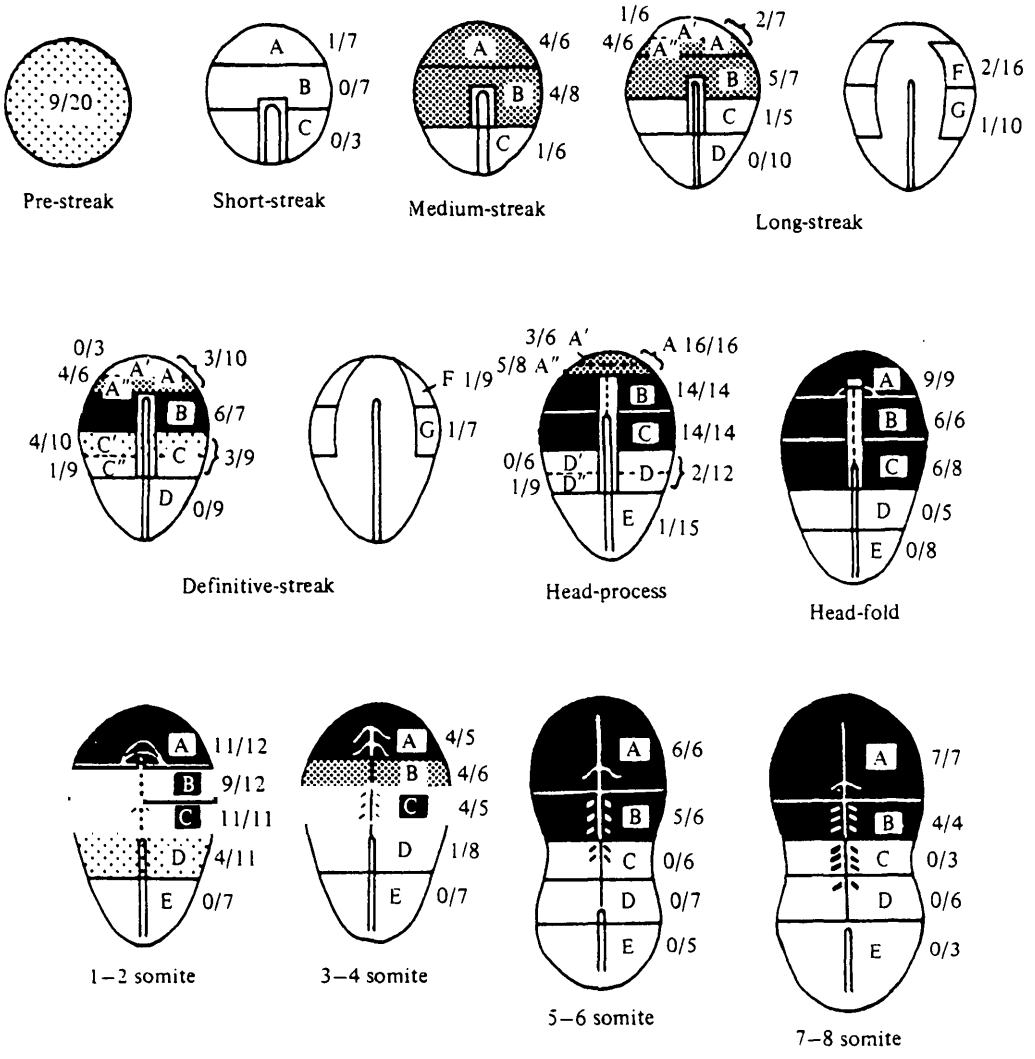


Fig. 1. Distribution of the pre-hepatic endoderm in the endoblastic layer. The ratio represents the number of the hepatic epithelial differentiation to the total number of explants. Median square areas in the short-streak to head-fold stages were excluded from the explants. Black, designates the areas where induced hepatic differentiation was from 75% up; dark shadow, from 50% up; light shadow, from 25% up; blank, under 25%.

the mesoblast but not endoblast. The primitive-streak region was excluded from the preparation of the epiblast.

Dissection of the primitive streak

The primitive streak, with some surrounding tissue, was cut from quail blastoderm at the short-streak to head-fold stage. The primitive streak was cut into three parts (anterior, middle and posterior) of equal size. The anterior part

of the primitive streak was further divided into upper (dorsal) and lower (ventral) halves.

Preparation of endodermal fragments

Endodermal fragments were obtained from quail embryos at the pre-streak to 8-somite stage.

At the pre-streak stage, whole hypoblast was isolated mechanically. At the short-streak to 8-somite stage, blastoderms were cut into pieces A to C or A to G as shown in Fig. 1. In some cases area A was further divided into areas A′ and A″, C into C′ and C″, and D into D′ and D″. Areas represented by the same symbols in different stages of blastoderms in Fig. 1 do not always express exactly equivalent area.

From each piece of the blastoderm, endodermal fragments were isolated by collagenase treatment. After separation, the endodermal fragments were thoroughly washed as described above. Isolation of endodermal fragments with other enzymes (trypsin or hyaluronidase) or without enzymes was not effective. The endoderm of the primitive-streak region at the short-streak to head-fold stage, head-process region at the head-process to head-fold stage, and oral plate region at the 1- to 8-somite stage was too adhesive to separate clearly from mesectoderm even in the presence of enzymes. Therefore, these regions were excluded from explants.

Isolation of inductive mesoderm

Cardiac mesoderm taken from the precardiac and cardiac region of 1·5-day (the 4- to 11-somite stages) chick embryos with collagenase treatment used as the inductor throughout the experiments.

Culture and graft

Pieces of blastoderms and cardiac mesoderm were associated *in vitro* on Wolff & Haffen's (1952) semi-solid culture medium for 1 day. The culture medium consisted of seven parts of 1 % of Difco Bacto-Agar in Gey's solution, three parts of foetal bovine serum (Flow Laboratories Ltd), three parts of Medium 199 and one part of Tyrode's solution containing potassium penicillin G. Explants were then transplanted into the coelomic cavity of 3-day chick embryos and cultivated further for 6 days. For controls, fragments of blastoderms alone were cultured *in vitro* and transplanted into the coelomic cavity.

Histological methods

Explants were fixed in Bouin's fluid, embedded in paraffin, sectioned at 5 μm and stained with haematoxylin and eosin.

Table 1. *Differentiation of the hepatic epithelium from various fragments of epiblast and primitive streak cultured with or without cardiac mesoderm*

Areas of the epiblast and primitive streak			Stages			
			Unincubated	Pre-streak	Short-streak	Long- to Definitive-streak	Head-process to Head-fold
			X–XII (Eyal-Giladi & Kochav)	1 (Vakaet)	3 (Vakaet)	5–6 (Vakaet)	8–9 (Vakaet)
Epiblast	Anterior		4/17* (3/6)	3/16† (6/14)	0/6	0/4	—
	Posterior				1/9	0/2	—
Primitive streak	Anterior	Upper half			10/14 (1/11)	2/12 (0/13)	0/7 (0/6)
		Lower half			8/12 (0/5)	9/13 (0/10)	1/8 (0/5)
	Middle				2/10	1/9	—
	Posterior				1/9	0/5	0/2

—, Not examined.
Number designates the hepatic differentiation to the total number of explants.
Number in parentheses designates the hepatic differentiation when the cardiac mesoderm was not associated. *, Whole blastoderm was used. †, Whole epiblast was used.

Identification of pre-hepatic cells

A fragment of quail blastoderm was always associated with chick cardiac mesoderm to show the origin of the differentiated tissues after cultivation (Le Douarin, 1969). Fragments of blastoderms which differentiated into hepatic epithelium under the inductive influence of the cardiac mesoderm were considered to contain pre-hepatic cells. The differentiation of the hepatic epithelium was identified by the presence of bile canaliculi and the formation of hepatic cords.

Though the size of fragments of blastoderms differed to some extent according to region, each fragment used in the present study was large enough to elicit hepatic induction, since hepatic epithelium was frequently induced, even in the smallest fragments of the lower half of the anterior area of the primitive streak in the short-streak-stage embryo.

RESULTS

The distribution of the pre-hepatic cells was investigated by examining differentiation into hepatic epithelium, when a fragment of the epiblast, primitive-streak or endoblastic layer was cultured under the inductive stimulus of cardiac mesoderm.

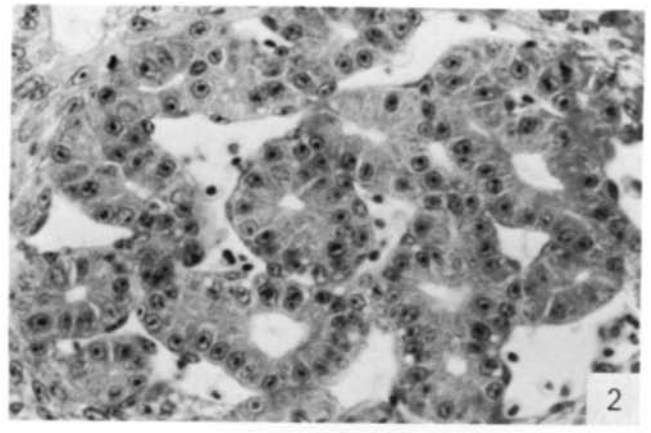
(1) *Pre-hepatic cells in the epiblast*

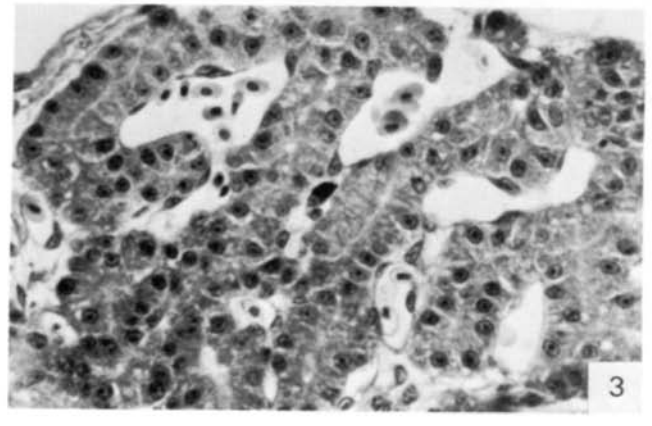
Pre-hepatic cells were detectable in the whole blastoderm or whole epiblast of unincubated or pre-streak stage. (Table 1 and Fig. 2). However, hepatic differentiation was also observed in pieces of blastoderms cultured alone without cardiac mesoderm, suggesting that this is due to the presence of precardiac cells or their progenitors in the blastoderm itself. From the short-streak stage on, pre-hepatic cells disappeared from the epiblast.

Endodermal epithelia other than the hepatic epithelium, such as yolk-sac, oesophageal, proventricular, gizzard, pancreatic and small intestinal epithelia were differentiated from the epiblastic explants up to the short-streak stage, but not from those older than the long-streak stage, irrespective of the presence of cardiac mesoderm. In contrast, neural structures and mesodermal derivatives such as notochord, cartilage and muscle were differentiated from epiblastic explants of all stages at least up to the definitive-streak stage.

(2) *Pre-hepatic cells in the primitive streak*

When pieces of primitive streak alone were cultivated, the hepatic epithelium hardly differentiated (Table 1). Pre-hepatic cells appeared in the anterior part of the primitive streak just before the gastrulation (short-streak stage). They were present in both the upper and lower half of the anterior part of the primitive streak (Table 1). Pre-hepatic cells, however, disappeared almost completely





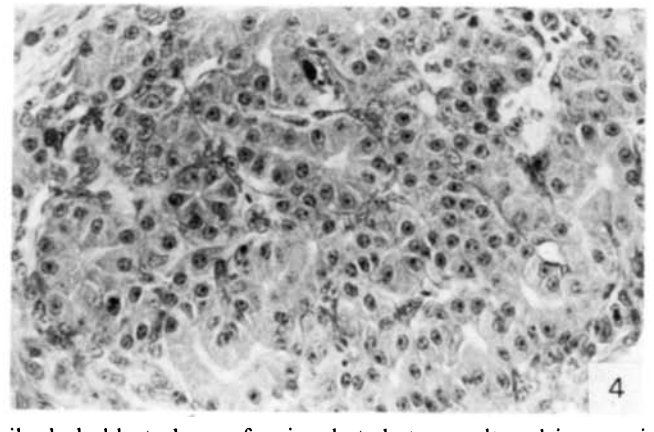


Fig. 2. Quail whole blastoderm of unincubated stage cultured in association with chick cardiac mesoderm for 6 days in the coelomic cavity. Differentiation of the quail hepatic epithelium forming hepatic cords and bile canaliculi. × 300.

Fig. 3. Quail hypoblast of pre-streak stage cultured in association with chick cardiac mesoderm for 6 days in the coelomic cavity. Quail hepatic epithelium was differentiated. × 300.

Fig. 4. Endodermal fragment anterior to the Hensen's node (area A″ in Fig. 1) of the long-streak stage quail embryo cultured in association with chick cardiac mesoderm for 6 days in the coelomic cavity. Differentiation of the quail hepatic epithelium. × 250.

from the upper half during subsequent stages of gastrulation (the long- to definitive-streak stages) and concentrated in the lower half. At the head-process and head-fold stages, pre-hepatic cells were no longer demonstrated in the primitive streak. In the middle and posterior parts of the primitive streak, pre-hepatic cells were rarely present irrespective of stage, suggesting that the pre-hepatic cells invaginate through the anterior part of the primitive streak during the gastrulation.

Other types of endodermal epithelia such as oesophagus, proventriculus and small intestine were differentiated in the explants of upper half of the anterior part of the primitive streak up to the definitive-streak stage, and in those of the lower half at all stages examined. From the middle and posterior parts of the primitive streak, no endodermal epithelia except the yolk-sac epithelium differentiated. From the explants of upper and lower halves of the anterior part of the primitive streak, not only endodermal epithelia but also neural structures, notochord, cartilage, muscle and mesonephros differentiated.

(3) *Pre-hepatic cells in the endoblastic layer*

According to percentages of hepatic differentiation, areas of the endoblastic layer were graded into four classes (Fig. 1).

The hypoblast of the pre-streak stage could differentiate into the hepatic epithelium when associated with cardiac mesoderm (Fig. 3), but could not when cultured alone (0 cases out of 9). In contrast, the hypoblast of the short-streak stage no longer differentiated into the hepatic epithelium even in the presence of the cardiac mesoderm.

From the medium-streak stage on, the pre-hepatic cells are located in the endoblastic layer (Fig. 1). At the medium-streak stage, they exist in the anterior two-thirds of the endoblast.

By the long-streak stage, the pre-hepatic endodermal area moved to the central area of the anterior half of the endoblast (Figs. 1, 4).

Pre-hepatic endoderm became concentrated at the level including Hensen's node (area B in Fig. 1) at the definitive-streak stage, but it also extended anteriorly and posteriorly to area B (areas A″ and C′). However, pre-hepatic endoderm was not found in the anterior (area A′) and lateral periphery (areas F and G) of the blastoderm.

The endoderm of the level including the head-process, Hensen's node and the anterior part of the primitive streak (areas B and C) contained pre-hepatic endoderm at the head-process stage. At this stage pre-hepatic endoderm was also detected in the areas A′ and A″ though the incidence of hepatic differentiation was lower than those in the areas B and C.

At the head-fold stage, the entire anterior half of the endoderm (areas A, B and C) included pre-hepatic endoderm. In addition to the anterior half of the endoderm (areas A, B and C), pre-hepatic endoderm occasionally existed in the anterior part of the posterior half endoderm (area D) at the 1- to 2-somite stage.

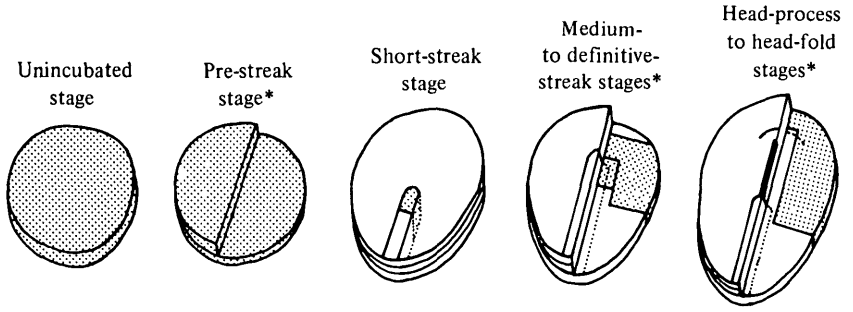


Fig. 5. Distribution of the pre-hepatic cells or pre-hepatic endoderm between the unincubated and head-fold stages of quail blastoderms. Dotted area represents the presence of the pre-hepatic cells. *, Right side of each figure shows endoblastic layer.

From the 3-somite stage on, the pre-hepatic and non-hepatic areas were clearly separated at the level of the 3rd somite (Fig. 1).

The movements of the pre-hepatic cells during early developmental stages of the quail blastoderm are illustrated in Fig. 5. The dotted area designates the pre-hepatic area. During unincubated and pre-streak stages, whole blastoderm can respond to the inductive influence of the cardiac mesoderm. At the short-streak stage, just before the beginning of gastrulation, the pre-hepatic cells are compressed in the anterior part of the primitive streak. When gastrulation advances during the medium- to definitive-streak stages, pre-hepatic cells appear in the endoblastic layer. They are also present in the lower half of the anterior part of the primitive streak. After gastrulation, the pre-hepatic area is restricted to the anterior half of the endoderm. From the 1-somite up to the 8-somite stages, the pre-hepatic area is consistently localized in the endoderm, anterior to the level of the 3rd somite.

DISCUSSION

The distribution of cells which can react with the cardiac mesoderm and differentiate into hepatic epithelium (pre-hepatic cells) was followed in quail embryos between the unincubated and 8-somite stages. These cells first appear in the blastoderm of unincubated stage, concentrate in the anterior part of the primitive streak and invaginate through this area of the primitive streak during the gastrulation. When gastrulation proceeds, pre-hepatic cells appear in the endoblastic layer. After gastrulation is completed, they become restricted to the anterior half of the endoderm.

By culturing whole blastoderms of definitive streak to 11-somite-stage chick embryos on the chorioallantoic membrane (CAM), Willier & Rawles (1931*a*, *b*) first reported the differentiation of hepatic tissues. Hunt (1931, 1932), Rudnick (1932) and Rawles (1936) located the hepatogenic regions in the chick embryo of the definitive streak to head-fold stages, using the technique of

CAM-grafting of fragments of blastoderms including three germ layers. These results define the areas containing both the pre-hepatic endoderm and the inductive mesoderm, but areas containing only the pre-hepatic endoderm cannot be detected by this method.

Hunt (1937) separated blastoderms into mesectodermal and mesentodermal layers. He transplanted fragments of these two layers separately onto CAM and found that hepatic tissues develop from mesectodermal fragments including the anterior part of the primitive-streak up to the long-streak stage. The hepatic tissue, however, rarely develops from the mesectoderm posterior to the primitive pit and lateral to the primitive streak. In the present study, it is shown that pre-hepatic cells are not in the mesectoderm after primitive-streak formation except for the anterior part of the primitive streak. Therefore, hepatic differentiation from the mesectoderm observed by Hunt (1937) might be attributed to the anterior part of the primitive streak present in his material.

The individual locations of pre-hepatic endoderm and mesoderm were first demonstrated by Le Dourin (1964*a*) with chick embryos. Using carbon marking experiments, radiodestruction of transverse levels of embryos behind the heart rudiment, and *in vivo* transplantation of fragments of embryos she concluded that the pre-hepatic endoderm is restricted to positions anterior to the level of the 1st somite. In the present study, however, the pre-hepatic endoderm was also found in endodermal fragments at the level of the 1st to 3rd somite (13 cases out of 15, Fig. 1) and 2nd to 4th somite (8 cases of 15, data not shown) in the 3- to 10-somite-stage quail embryos. Since pre-hepatic endoderm was rarely found in endodermal fragments at the level of the 3rd to 5th (1 case out of 8, data not shown) and 4th to 6th (1 cases out of 17, Fig. 1) somite, the transverse boundary between the pre-hepatic and non-hepatic endoderm is between the 2nd and 3rd somite in the quail embryo.

Though the quail blastoderm–chick cardiac mesoderm system was used in the present study, the induction itself is not due to the heteroplastic interaction, since hepatic induction takes place in chick blastoderm cultured in a homoplastic environment.

By radioautographic mapping, Rosenquist (1971*a*) investigated the localization of the pre-hepatic endoderm and mesoderm. The distribution of the pre-hepatic endoderm in the endoblastic layer coincides well with the results of the present study, except that he also detected pre-hepatic endoderm posterior to the 3rd somite. The criterion used for pre-hepatic endoderm by Rosenquist was incorporation into the hepatic primordium, whereas we define it by its ability to differentiate into hepatic epithelium under the influence of cardiac mesoderm.

In the present study it was demonstrated that during the short- to definitive-streak stages, pre-hepatic cells invaginate through anterior third of the primitive streak, and that, during the medium-streak to head-fold stages, they are restricted to the anterior half of the endoblastic layer, centering around the tip of the primitive streak or head process. Both the timing of invagination of pre-

hepatic cells and their distribution in the endoblastic layer, coincide well with the definitive endoblast which invaginates from the epiblast through Hensen's node during the short- to definitive-streak stage (Nicolet, 1965, 1970; Rosenquist, 1966; Gallera & Nicolet, 1969; Vakaet, 1970) and extends in a concentric manner within the endoblastic layer (Vakaet, 1970; Rosenquist, 1971*b*). Therefore during invagination, the pre-hepatic cells move together with the definitive endoblast.

In contrast, precardiac mesoderm does not invaginate through the node but invaginates through the middle part of the primitive streak during the early short-streak to head-process stages (Nicolet, 1970; Rosenquist, 1970). Therefore, pre-hepatic cells and the precardiac mesoderm are considered to be separate, at least during gastrulation.

Precise comparison between the invagination of the pre-hepatic cells and of the endoblast lead us to conclude that invagination of the endoblast continues after completion of invagination of pre-hepatic cells, i.e. endoblast cells still exist in the epiblast at the short-streak stage, where the pre-hepatic cells cannot be found. The pre-hepatic cells have run through the upper and lower half of the anterior part of the primitive streak (Hensen's node) at least by the long-streak and head-process stage respectively, whereas endoblast cells still differentiate from the upper and lower half of Hensen's node up to the definitive-streak and head-fold stages respectively. Veini & Hara (1975) reported that endoblast cells exist in the lower layer of Hensen's node at least up to the head-fold stage. These observations suggest that the pre-hepatic cells are already determined or may be grouped together but separating from the definitive endoblast, when they invaginate through the Hensen's node.

After invagination, the pre-hepatic endoderm becomes restricted in the anterior half of the definitive endoblast. This may explain why the endoderm which has hepatogenic potency develops from the anterior half but not from the posterior half of the embryo (Fukuda, 1979). However, the mechanisms of restriction of the pre-hepatic endoderm to the anterior half of the definitive endoblast are not known. Gradual shift of the invagination centre of the definitive endoblast within the primitive streak (Vakaet, 1970) may be one of the reasons.

The hypoblast contributes to the formation of the extraembryonic endoderm but not of the embryonic endoderm (Modak, 1966; Rosenquist, 1966; Nicolet, 1970; Vakaet, 1970; Wolk & Eyal-Giladi, 1977). In the present study, hepatogenic potency was also found in the hypoblast of the pre-streak-stage embryos. At this stage, the presumptive definitive endoblast is present in the epiblast and the invagination of the definitive endoblast has not yet begun. Since the hypoblast can be easily separated from the epiblast, contamination of presumptive definitive endoblast in the 'hypoblast' used in the present study is unlikely. Recently, the extensive ability of extraembryonic endoderm to respond to various stimuli of inductive mesoderms such as digestive tract mesoderms has

been demonstrated (Masui, 1981). That the hypoblast of pre-streak stage can also respond to the inductive stimulus of the cardiac mesoderm may be considered. However, the reactivity to the cardiac mesoderm disappears from the hypoblast at the short-streak stage and pre-hepatic cells invaginate through the primitive streak at later stages. Therefore, the hypoblast of the pre-streak stage which can react with the cardiac mesoderm cannot be considered as a proper pre-hepatic endoderm.

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