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Avian marginal zone cells function as primitive streak inducers only after their migration into the hypoblast

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SUMMARY

Hypoblast cells of posterior marginal zone origin have been shown previously to be the inducers of primitive streak in the avian embryo. Here we checked: (1) whether the above cells acquire their inductivity while still whithin the marginal zone; (2) can inductivity be found in supernatants of defined blastodermic regions; (3) can differences in the electrophoretic pattern be shown between inducing and non-inducing tissue fragments and their conditioned media, which might give a clue as to what the inductive substance is.

The following observations were made:

1. (a) Stage X chick posterior marginal zone cells prior to their migration into the hypoblast do not induce a primitive streak, when applied to a stage XIII competent epiblast central disc. (b) A posterior marginal zone fragment, when applied to an epiblast central disc, even after being preincubated for up to 9 hours in vitro, is still non-inductive. (c) Mechanically fragmented stage X posterior marginal zones when applied as a layer to epiblast central discs are non-inductive. (d) Hypoblastic tissue in strip form induces a primitive streak.

2. Competent stage XIII epiblast central discs (chick) were incubated for 2 hours in supernatants of stage XIII epiblasts or hypoblasts. Whereas no inductive effect was exerted by the epiblast supernatant, primitive streaks developed in about 50% of the epiblast central discs incubated in the hypoblast supernatant.

3. Electrophoretic analysis (quails) reveals a protein of 28×10^{-3} M_r that is enriched in both hypoblastic tissue and its incubation medium and not in the epiblast + marginal zone + area opaca and their incubation medium. These findings suggest a possible correlation between this protein and the induction process.

Key words: avian marginal zone, primitive streak induction, hypoblast, electrophoretic study

INTRODUCTION

A stage X EG&K (Eyal-Giladi and Kochav, 1976) unincubated chick blastoderm has the shape of a thin disc of cells about 2 mm in diameter. It can roughly be subdivided into two circular areas: the thick peripheral area opaca and the transparent, mainly single-layered, central disc - the area pellucida. However, in some blastoderms it is already possible to distinguish, a crescent-shaped ridge on the ventral surface of the epiblast, Koller's sickle, which is situated at a certain distance, centralwards to the posterior inner margin of the area opaca (Fig. 1). The demarcation of the crescent shaped strip of the area pellucida located between Koller's sickle and the area opaca, is the first indication of the gradual appearance of the circular marginal zone belt. The marginal zone is single layered and the epiblast in front of Koller's sickle is its direct continuation. During the first 10 hours of incubation, a hypoblast grows and spreads underneath the epiblast in a posteroanterior direction with Koller's sickle being its posterior limit. The fully grown hypoblast (stage XIII EG&K) thus covers only the central area of the lower surface of the epiblast, leaving around it a clearly demarcated, peripheral single-layered marginal zone, which separates the two-layered central disc from the thick peripheral area opaca.

The formation of the hypoblast was shown to be a complex process and the cells from which it is formed are claimed to derive from at least two different sources. One source is the polyingressing cells coming down from the epiblast, while the other source includes the cells of marginal zone origin that have advanced anteriorly via Koller's sickle (Vakaet, 1962; Eyal-Giladi and Kochav, 1976; Eyal-Giladi, 1992; Eyal-Giladi et al., 1992). The two cell populations merge in the final confluent hypoblastic sheet where they are indistinguishable from one another.

The hypoblast has been shown to be responsible for the induction of the primitive streak (Waddington, 1933; Eyal-Giladi and Wolk, 1970; Azar and Eyal-Giladi, 1981; Mitrani and Eyal-Giladi, 1981, 1984). Azar and Eyal-Giladi (1979), on the basis of elimination experiments, came to the conclusion that only hypoblastic cells of marginal zone origin and not of polyingression origin, are capable of inducing a primitive streak in the epiblast. The above conclusion was substantially supported by the work of Khaner and Eyal-Giladi (1989) and Eyal-Giladi and Khaner (1989) who showed that the point of origin of the primitive streak could be shifted laterally by transplanting, in a stage X blastoderm, the most posterior section of the marginal zone, into a lateral position within the marginal zone.

Recently we have shown by fluorescent labelling (Eyal-Giladi et al., 1992), that labelled cells of posterior marginal zone origin indeed move via Koller's sickle into the forming hypoblast, in which they occupy a central position. There they are surrounded laterally by non-labelled hypoblastic cells probably from polyingression origin.

In the present study we have investigated the inductive potency of the posterior marginal zone, as compared to that of the hypoblast in order to find out:

(1) When the cells of marginal zone origin acquire their inductivity; is it prior to, or only after their migration into the hypoblast.

(2) Could a primitive streak be induced by supernatants of incubated hypoblasts or epiblasts.

(3) Would it be possible to trace a natural substance suspected of being the inducer of the primitive streak.

MATERIALS AND METHODS

For experimental series I and II chicken eggs were used, while quail eggs were used for series III (the electrophoretic study).

Preparation of posterior marginal zone fragments

Eggs were collected shortly after laying. Stage X blastoderms in which Koller's sickle was clearly identified were used as posterior marginal zone donors. The posterior marginal zones were cut out either with or without Koller's sickle (Fig. 1).

Preparation of epiblasts and hypoblasts for manipulations

Stage XIII blastoderms were used for preparing epiblastic central discs in the following way (Fig. 1): a circular cut was made somewhat central to the peripheral border of the full hypoblast, thus separating the central disc from the marginal zone and area opaca, which were removed. The cut was made so that at the posterior side the distance from the peripheral border of the hypoblast towards the center was larger than that at the anterior side. This was done to avoid any contamination of either the epiblastic disc, or the hypoblast, with cells that might concentrate between the two at the posterior side. Afterwards, the hypoblast of the central disc was either removed or collected for further experimental use.

The exposed ventral surfaces of the epiblastic central discs were used as recipients for the tested tissues. During the collection period the fragments were kept on ice, which was shown not to interfere with their developmental potencies.

A solid culture medium of the following composition was used (Mitrani and Shimoni, 1990). 200 mg of low melting point agarose (IBI) were dissolved in 3 ml H_2O and autoclaved. For the cooling off, agarose was mixed with 8 ml RPMI (Roswell Park Memorial Institute), 10 μl penicillin and 100 μl glutamine and immediately poured into 35 mm Petri dishes. This medium was used to culture the epiblastic central disc fragments for 48 hours, after their treatment with the supernatants.

Stage XIII (EG&K) quail blastoderms were used for electrophoresis. The blastoderms were subdivided with a hair loop into the three desired components, namely: epiblast, hypoblast and area opaca + marginal zone. The amount of protein in the samples used for the following experiments was determined according to the method of Pesce and Strande (1973). The average protein content of a stage XIII quail's blastoderm is 57 μg. The distribution among the three experimental fragments, epiblast, hypoblast and area opaca + marginal zone is 21.5% (12 μg), 15.7% (8.7 μg) and 62.8% (35 μg) respectively.

Preparation of incubation media

Various numbers of stage XIII hypoblasts were pooled together and transferred to Eppendorf test tubes containing 50 μl of Ringer's solution. The test tubes were coated with bovine serum albumin (BSA) to avoid a possible absorption of inductive molecules into the plastic.

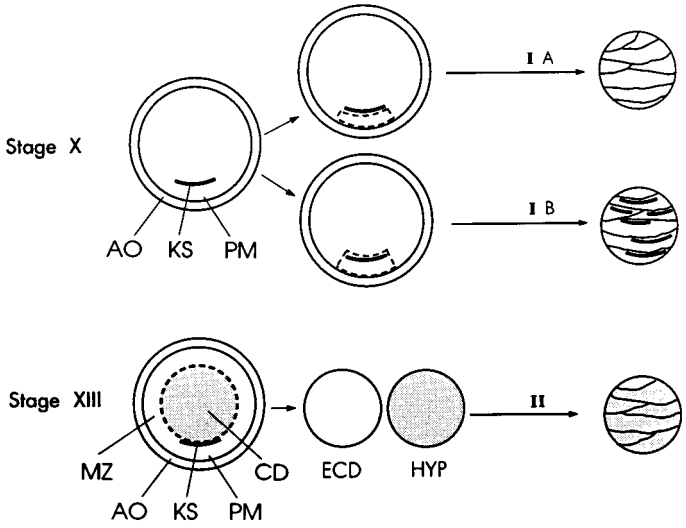

Fig. 1. Experimental procedure. Stage XIII epiblastic central discs were used in all experiments as the reacting system. Stage XIII epiblastic central discs, hypoblasts, and stage X posterior marginal zones with or without Koller's sickle were tested as potential inducers. IA: posterior marginal zones without Koller's sickle were arranged on an epiblastic central disc to cover its entire ventral surface. IB: the same procedure was done with posterior marginal zones containing a Koller's sickle. II: a similar procedure was done with hypoblast strips, without paying attention to their original axis or original location in the hypoblast. AO, area opaca; CD, central disc; ECD, epiblastic central disc; HYP, hypoblast; KS, Koller's sickle; MZ, marginal zone; PM, posterior marginal zone.

The tubes containing the hypoblasts were incubated at 37°C for 2.5-3 hours, and were occasionally shaken. The tubes were centrifuged for 5 minutes at 14,000 rpm and the supernatants were removed to other test tubes (coated with BSA). For each test tube the F value was calculated, which specified the number of hypoblasts per volume of Ringer's solution. (F = number of hypoblasts per μl Ringer's solution).

For a control experiment, epiblasts were treated similarly and their supernatants were prepared according to the same procedure as for the hypoblast. F values were also similarly calculated for each test tube. The tubes with the supernatants were frozen at –20°C and then thawed at room temperature before use.

Several previously prepared epiblastic central discs were placed into test tubes with thawed supernatants of a known F value, and incubated at 37°C for 2 hours. After the incubation, they were taken out, rinsed in Ringer's solution and cultured for 48 hours on plates containing 1 ml of solid culture medium.

[^{35}S]methionine labelling

Fragments of each of the three regions from 10 different quail blastoderms were pooled and immersed in test tubes containing 0.5 ml Dulbecco's modified Eagle's medium, the methionine of which was replaced by 50 μCi [^{35}S]methionine (1340 Ci/mmol Amersham SJ. 204) and was further incubated for 90 minutes at 37°C (Steinberg 1983). The incorporation of radioactive methionine was terminated by the addition of cold phosphate-buffered saline (PBS), pH 7.2. After centrifugation for 10 minutes at 1000 rpm the incubation media were stored at –70°C, while the radioactive tissue was washed several times with cold medium and then lysed in buffer containing: 10 mM $NaHPO_4$, pH 7.5, 100 mM NaCl, 1% Triton X-100, 0.5% deoxy-

Table 1. Development of primitive streaks in competent stage XIII epiblasts after interaction with either posterior marginal zone or hypoblastic fragments

		I. Posterior marginal zone fragments applied to stage XIII epiblastic central discs			II. Hypoblastic fragments applied to stage XIII epiblastic central discs			
		No. of experiments	No. of fragments	% PS developed	No. of experiments	No. of fragments	% PS developed	
Ventral surface facing the epiblast	with KS	11	3-6	0	13	5-15	84.6	
	w/o KS	10	3-7	0				
Dorsal surface facing the epiblast	with KS	11	3-6	0	-	-	-	
	w/o KS	11	3-9	0				
Fragmented posterior marginal zones	w/o KS	10	3-8	0	-	-	-	
Only posterior thirds					5	2	100	94.7
					14	3	93	
Only anterior thirds					5	2	80	52
					14	3	43	

KS, Koller's sickle.
PS, Primitive streak.
w/o, without.

cholate, 0.1% SDS and 1% aprotinin (Sigma Chemical Co., St. Louis, Mo.), centrifuged at 14000 rpm for 5 minutes and the supernatant containing the cell lysate stored at −70°C.

Gel electrophoresis

The extracts from each of the three blastodermic components containing the equivalent of 300,000 TCA precipitable counts were exposed to SDS polyacrylamlide gel electrophoresis (PAGE) according to the method of Laemmli (1970) with a 5% stacking gel and a 10% separating gel. The incubation medium from each fragment was first precipitated with 7.5% TCA and then exposed to electrophoresis. 69,000 TCA precipitable counts were used per lane. Fluorography of the radiolabelled samples was done according to the method of Bonner and Laskey (1974). Scanning of the autoradiograms was done with a Zemeh soft laser scanning densitometer model SL-TRFF 10.

EXPERIMENTAL PROCEDURES AND RESULTS

Series I: inductivity of posterior marginal zone versus hypoblast

Chick stage XIII competent epiblastic central discs, were used, which have been shown in earlier studies to be competent to react to inductive stimuli by forming a primitive streak (Azar and Eyal-Giladi, 1981; Mitrani and Eyal-Giladi, 1981, 1984). To the ventral side of the epiblastic central discs, the following tissues were applied: strips of stage XIII hypoblasts of different locations and combinations, stage X posterior marginal zones with and without Koller's sickle; stage X fragmented posterior marginal zones, and stage X posterior marginal zones after preincubation for several hours. All the different sandwiches were incubated for 24-48 hours, on a vitelline membrane (New, 1955) after which they were checked for axis formation.

Controls

Before starting to check the effect of posterior marginal zones on competent stage XIII epiblastic central discs, we did two control experiments to verify again the reaction of the epiblastic central discs to an implanted hypoblast, as compared to implantation of another epiblastic central disc.

(1) Seven epiblastic central discs were cut out and at the same time 7 hypoblasts were prepared. The anteroposterior axis was marked with carbon and carmine particles on both the epiblastic central disc and the hypoblast. The ventral surface of each disc was covered with a hypoblast from another stage XIII blastoderm, with the anteroposterior axes of the two coinciding. In all the cases a primitive streak developed from the disc, according to its correct polarity (Fig. 2A).

(2) 16 epiblastic central discs were each covered with a similar disc from another stage XIII blastoderm. The ventral sides of both discs faced each other and their anteroposterior axes coincided. No primitive streak developed in any of these epiblast sandwiches.

The inductivity of stage X posterior marginal zones

Several stage X blastoderms with a distinct Koller's sickle were collected. From each blastoderm the posterior marginal zone, which did not include the area opaca, was cut out. Groups of posterior marginal zone strips, with or without Koller's sickle, were collected separately. The length of each strip was equal to the length of Koller's sickle and its width equalled the entire marginal zone from the inner border of the area opaca to Koller's sickle. Several posterior marginal zone strips, sufficient for one experiment, were collected into a dish and kept at 4°C to prevent further development. A previously set-aside stage XIII recipient blastoderm was then prepared, by removing its hypoblast and thus exposing the ventral surface of the epiblast. The area opaca plus marginal zone were removed. The posterior marginal zone strips were transferred into the glass ring containing the recipient epiblastic central disc and aligned side by side to cover its entire ventral surface. The orientation of the posterior marginal zone strips was arbitrary in relation to the anteroposterior axis of the recipient (Table 1 I). The experimental material was then incubated for 48 hours. The marginal zone fragments healed very quickly to form a single coherent sheet.

Posterior marginal zone without Koller's sickle (Fig. 1 IA)

(1) In ten experiments the posterior marginal zone strips were put with their ventral surface facing the ventral surface of the epiblastic central disc (Fig. 2B).

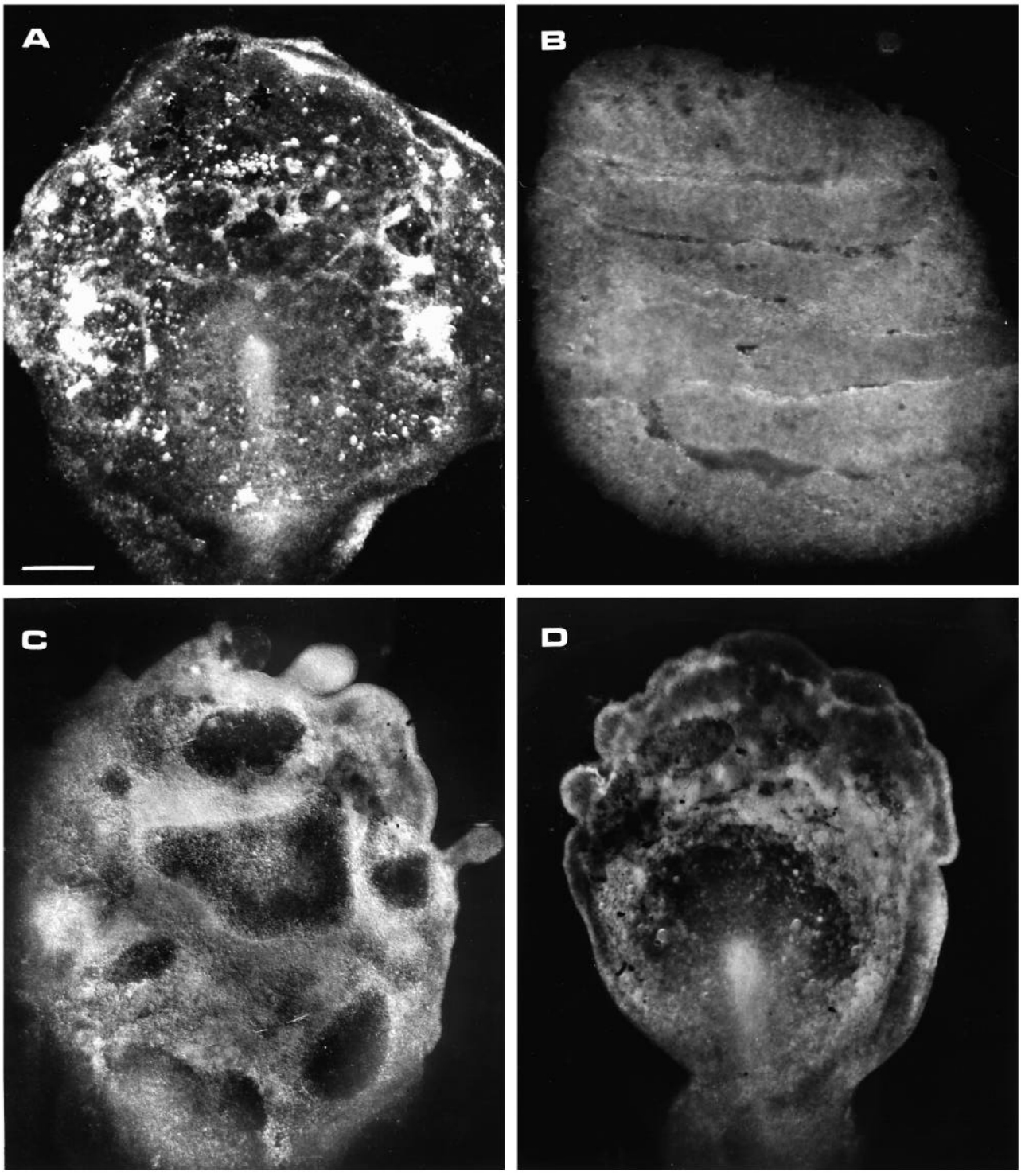

Fig. 2. (A) A stage XIII epiblastic central disc was covered with a stage XIII hypoblast from a different blastoderm with the anterioposterior axis of the two coinciding. A single primitive streak developed in the correct polarity. (B) A stage XIII epiblastic central disc immediately after being covered with posterior marginal zone strips. (C) Same as B after 48 hours of incubation. No primitive streak developed. (D) A stage XIII epiblastic central disc was covered with several posterior fragments of stage XIII hypoblasts. After 48 hours of incubation a single primitive streak formed according to the original polarity of the epiblast. Scale bar, 0.5 mm.

(2) In eleven experiments the posterior marginal zone fragments were aligned with their dorsal surface facing the ventral surface of the epiblastic central disc.

(3) In ten experiments the posterior marginal zones were mechanically fragmented with the help of steel needles into a mixture of small cell clusters and single cells. The fragmented tissue was spread over the entire ventral surface of the epiblast in a dense layer.

The rest of the fluid was then sucked out of the ring and the experimental material incubated for 40-48 hours, to check for PS development.

All the 31 experiments of the above three subgroups failed to form a primitive streak (Fig. 2C).

Posterior marginal zones with Koller's sickle (Fig. 1 IB)

Similar experiments as above, were performed using posterior marginal zone strips from which Koller's sickle was not removed. No primitive streak was seen to develop in any of the 22 experimental epiblastic central discs.

The inductivity of hypoblast strips (Fig. 1 II)

(1) Stage XIII hypoblasts were removed from the blastoderm and cut into strips similar in size to the posterior marginal zone strips in the previous experiments. About 5-15 strips from several hypoblasts were used to cover the ventral surface of one stage XIII epiblastic central disc, in a way similar to the experiments with the posterior marginal zone strips. In 11 out of 13 central discs (84%), which showed good development, a single primitive streak developed according to the anteroposterior polarity of the recipient central disc.

(2) The anteroposterior polarity of isolated hypoblasts was marked and each hypoblast was cut into 3 transverse strips. The median strips were discarded while the anterior and posterior strips were collected into two separate groups. Stage XIII epiblastic central discs were prepared as recipients and their ventral surface was covered with hypoblast strips. Equal numbers of discs were covered by either anterior hypoblast fragments or posterior hypoblast fragments. The number of hypoblast fragments varied between two and three depending on their size (Table 1 II):

(a) The ventral surface of 5 epiblastic central discs was covered with 2 hypoblast posterior thirds, while for 14 other discs it took 3 posterior hypoblast strips to cover the entire surface.

(b) The ventral surface of 5 discs was covered with 2 anterior hypoblast thirds, while in 14 additional discs the surface was covered with 3 anterior hypoblast fragments. In all the cases in which a primitive streak developed from the epiblastic central disc it was a single primitive streak according to the previously marked anteroposterior polarity of the epiblast.

A significant difference was observed between groups a and b. While in group a, with the posterior fragments, a primitive streak developed in 94.7% of the cases (Fig. 2D), in group b, with anterior fragments, only in 52% of the experiments did a primitive streak develop. Furthermore there is also an indication of a remarkable difference between the two subgroups of group b. While in the experiments with only two anterior hypoblast fragments a primitive streak developed in 80% of the epiblastic central discs, only 43% of the epiblastic discs formed a primitive streak when three anterior fragments were needed to cover the ventral surface of the disc.

The inductivity of preincubated posterior marginal zones

A supplementary series of experiments was done to check whether the age difference between the epiblastic central discs (stage XIII) and the posterior marginal zone implants (stage X) might have been responsible for the negative results. The possibility existed that a stage X marginal zone implant might need a few more hours of incubation to become inductive and in the meantime the stage XIII recipient central disc might lose its short lived competence to form a primitive streak (Azar and Eyal-Giladi, 1981). We therefore incubated the isolated marginal fragments on a vitelline membrane for different lengths of time (Table 2), prior to their implantation onto the central disc. A single incubated posterior marginal zone was then applied to every central disc. In total, 32 such experiments were done and only in four of them was a small condensation of cells, which resembled an initial primitive streak, observed.

Table 2. The inductivity of preincubated posterior marginal zones

Hours of incubation of posterior marginal zone fragments prior to implantation	No. of experiments	% of rudimentary primitive streak-like thickenings
3-5	18	(2 cases) 11
5.5-6.5	6	(1 case) 16.5
7-9	8	(1 case) 12.5
Total	32	12.5

The posterior marginal zone fragment was incubated on a vitelline membrane for several hours, after which it was applied to a freshly prepared stage XIII epiblastic central disc.

Table 3. Three control series to the experiments in Table 2

Type of control	No. of experiments	% PSs
Posterior marginal zone fragment grown for 48 h on a vitelline membrane	6	0
A single posterior marginal zone fragment implanted on epiblastic central disc without preincubation	7	0
A single hypoblastic fragment implanted without preliminary incubation	7	71.5

PS, primitive streak.

Three control series to the posterior marginal zone preincubation experiment were also done (Table 3).

(1) A single unincubated posterior marginal zone was applied to each of seven epiblastic central discs.

(2) Six posterior marginal zone strips were each grown on a vitelline membrane to see whether they could form primitive streaks on their own. In both these series not even a single primitive streak developed.

(3) Single hypoblastic strips from stage XIII blastoderms, equal in size to the posterior marginal zone strips used in the other experiments, were applied to an epiblastic central disc in order to test whether a single strip that covered only part of the disc's surface was sufficient to induce a primitive streak. No attention was paid to the region (anterior or posterior) of the hypoblast from which the strips were taken. In 71.5% of the cases a clear primitive streak developed.

Series II: the inductive effect of supernatants

The hypoblastic and the epiblastic supernatants were found to act very differently from one another on stage XIII chick epi-

Table 4. The inductive effects of supernatants

Incubated in	Concentration factor (F-value)	No. of epiblastic central discs	No. discs with axis	% Axial development
Hypoblast	0.16	7	-	0
supernatant	0.2-0.23	15	8	53.3
	0.28-0.33	28	14	50
	0.36-0.4	32	16	50
	0.5-0.66	12	3	25
	0.84-0.92	12	5	41.5
Epiblast	0.2-0.3	15	0	0
supernatant	0.35-0.4	12	0	0
7.5% FCS in Ringer's solution	-	59	44	67.8
Ringer's solution	-	100	6	6

blastic central discs (Table 4). Central discs incubated for 2.5 hours in a hypoblastic supernatant of F values 0.2-0.4 (see Materials and methods), promoted embryonic development in about 50% of the cases. The epiblastic supernatant with a similar F value did not promote any axis formation in similar central discs following a 2.5-hour incubation. Two additional controls were made to test the adequacy of the method and especially the effect of the duration of incubation time in a liquid medium, on the competent epiblastic central discs.

(1) Epiblastic central discs were incubated for 2.5 hours in 7.5% fetal calf serum (FCS) in Ringer's solution, after which they were cultured as above.

(2) Epiblastic central discs were incubated for 2.5 hour in Ringer's solution and then cultured.

The results, summarized in Table 4, indicate that while the treatment with Ringer's solution did not induce a primitive streak, the FCS containing solution was found to induce primitive streaks in nearly 68% of the treated epiblastic discs, which was in agreement with empirical previous observations of E. Mitrani (personal communication).

Series III: electrophoretic study of tissue fragments versus their incubation media

To investigate the pattern of cellular proteins, each of the three above blastodermic quail fragments: epiblast, hypoblast and area opaca + marginal zone was incubated with [^{35}S]methionine and run in 10% SDS-PAGE (see Materials and methods).

Among the many proteins appearing in the autoradiogram, one protein of approx. 28×10^{-3} M_r that appeared in all three blastodermic fragments, was more prominent in the hypoblast fragment (Fig. 3A, arrow).

In order to determine whether there are certain proteins that are differentially secreted from the cells of a specific population into the surrounding medium, the incubation media of the three types of fragments were TCA precipitated and exposed to SDS-PAGE and autoradiography. Fig. 3B demonstrates that a difference was indeed found between the three incubation media, in the labelling intensity of a band of approx. 28×10^{-3} M_r. The band in the hypoblast medium was very prominent not only when compared to the same band in the other two media, but also to any other band in the three media.

Scanning of the autoradiograms revealed that the 28×10^{-3} M_r band constitutes at most 1-2% of the labelled proteins in the hypoblastic tissue, while in the incubation medium of the hypoblast this band constitutes about 45-50% of the labelled proteins. The quantitative ratio (of the 28×10^{-3} M_r band) between the hypoblastic tissue and the supernatant is approximately 1:3 respectively, as judged from the scanning.

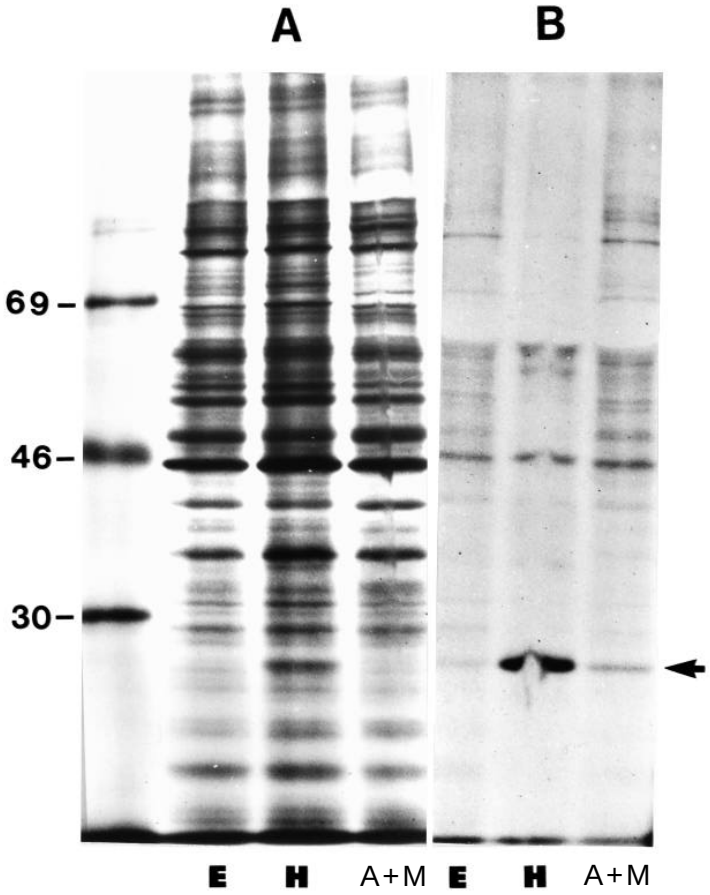

Fig. 3. Gel electrophoresis of quail blastodermic fragments and their incubation media. Fragments were metabolically labelled with [^{35}S]methionine as described in Materials and Methods. SDS-PAGE was performed for the fragments (A) and for the incubation media (B) of all three blastodermic fragments: epiblast (E), hypoblast (H) and area opaca + marginal zone (A+M). The arrow indicates the position of the 28×10^{-3} M_r band.

DISCUSSION

Several studies have indicated that the ability of a stage XIII EG&K hypoblast to induce a primitive streak in the overlying epiblast, stems from the cells of posterior marginal zone origin, which have populated the hypoblast (Azar and Eyal-Giladi, 1979; Khaner and Eyal-Giladi, 1989; Eyal-Giladi and Khaner, 1989; Eyal-Giladi, 1991, 1993; Eyal-Giladi et al., 1992). In a recent study Eyal-Giladi et al. (1992) show that, at stages X and XI, it is mainly cells located on Koller's sickle that will enter the hypoblast and probably become the inductive element for primitive streak. However, a stage XIII Koller's sickle and marginal zone are still capable to regenerate an inductive hypoblast if the original one is removed (Azar and Eyal-Giladi, 1979).

The question that we have tried to answer in the present

study was: are the cells of the posterior marginal zone inductive when still located in the marginal zone belt (Koller's sickle included) prior to their migration into the hypoblast, or do they acquire their inductivity only after that migration.

The reacting system in this study was a stage XIII chick epiblast central disc (cut out at a certain distance central to the marginal zone). Such discs have been shown by Azar and Eyal-Giladi (1979) to be incapable of forming a primitive streak when grown alone in vitro, but capable of forming a primitive streak when grown together with an overlying hypoblast. To rule out the possibility that the hypoblast did not function as a specific inductor, but rather as a lower layer, which permits the autonomous expression of an intrinsic ability of the epiblastic central disc to form a primitive streak, we applied another stage XIII epiblastic central disc to the lower surface of the discs as a substitute for the normal lower layer. While a sandwich of an epiblastic disc and a hypoblast mostly developed a primitive streak, a sandwich of two central discs never formed a primitive streak. This result confirmed that one can safely use the stage XIII epiblastic central disc as a reacting system to test the inductivity of stage X posterior marginal zones. The outcome of our experiments was clear cut and indicated that a posterior marginal zone fragment, when directly applied to the epiblastic central disc cannot induce a primitive streak in the epiblast (Table 1) even when the sandwich is incubated for 48 hours.

There are several possible explanations for this negative result as compared to the positive results with stage XIII hypoblasts. Firstly, the marginal zone cells, in order to acquire inductivity, have to be released from their epithelial packaging and actively migrate into the forming hypoblast. Secondly, at stage X the marginal zone cells are about 10 hours younger than a fully developed hypoblast and might therefore not yet have acquired the developmental maturity (dependent on an internal clock) to induce a primitive streak. If this is true then in the above sandwiches, the stage X posterior marginal zone cells might need several more hours of incubation to become inductive, while a stage XIII epiblastic disc, which is the reacting system, might in the meantime lose its competence to react.

Thirdly, the marginal zone cells need both ripening and movement in order to become inductive.

In the present paper we have checked some of the above possibilities in the following ways.

(1) The posterior marginal zone cells were released from their epithelial packaging by mechanical fragmentation (Table 1) and were immediately spread on the ventral surface of epiblastic central discs as small clusters and single cells. This, however, did not improve their state of non-inductivity, which again demonstrated that it is not the state of dispersion alone that causes posterior marginal zone cells to become inductive but something else or something in addition to their mechanical release from the rigid epithelial arrangement. In a similar experiment with trypsin and EDTA dissociated hypoblasts (Mitrani and Eyal-Giladi, 1981) primitive streaks were induced, as they were with whole hypoblasts. The relatively rough treatment did neither interfere with, nor enhance the inductivity of the hypoblastic cells.

(2) The experimental series in Table 2 checks the point of ripening. By incubating the posterior marginal zones on a vitelline membrane for up to 9 hours prior to their application to a stage XIII epiblastic central disc, both the central disc and posterior marginal zone were made roughly equally old. In that experiment, the epiblastic central disc is at its prime state of competence, and the posterior marginal zone cells (with Koller's sickle), which were prevented from migrating, continued to develop for a length of time that would normally bring them into the hypoblast. The negative results of this experiment rule out the factor of a 'generation gap' between the two as being a possible explanation for the negative results in series I, namely that the epiblastic central disc under our previous experimental conditions might have lost competence before the younger posterior marginal zone could have gained inductivity. It stresses the fact that the cells of the posterior marginal zone prior to migration, even when incubated for 9 additional hours, behave exactly as the cells of an epiblast and are non-inductive.

After showing that no change was achieved after the posterior marginal zone cells were released from their epithelial packaging or let ripen (10 hours of preincubation) before being applied to the epiblastic central disc, the importance of their active migration from the Koller's sickle into the growing hypoblast must be seriously considered.

One of the subjects dealt with in a recent paper by Izpisua-Belmonte et al. (1993), is the contribution of Koller's sickle to the formation of the primitive streak. The paper tries to promote the idea, earlier expressed by Stern and Canning (1990) that the primitive streak is not induced by the hypoblast as claimed by Waddington (1933), Eyal-Giladi and Wolk (1970), Azar and Eyal-Giladi (1981), Mitrani and Eyal-Giladi (1981) etc., but is formed of predetermined mesodermal cells that collect in the posterior side of the blastoderm in some kind of relation to Koller's sickle. To this purpose two sets of experiments were performed by Izpisua-Belmonte et al. In the first series, the lineage of Koller's sickle cells was studied by injection of DiI into the blastoderm close to Koller's sickle. The labelled cells were later found in Hensen's node and the conclusion was that they were the progeny of Koller's sickle. Injection of DiI is not a very precise method and in our opinion what happened in those experiments is that also the epiblastic cells dorsal to the sickle were labelled. The strip of epiblastic cells adjacent to the sickle has been shown by Eyal-Giladi et al. (1992) to be already induced to form the anterior section of the primitive streak at the stages used by the above authors. In another set of experiments, grafts of Koller's sickle were placed at 90° to the axis of the host and were claimed to induce an ectopic primitive streak composed of host-derived cells. According to the authors, the grafts contained a layer of hypoblast cells which held the sickle's cells together. In our opinion this was enough not only to induce a primitive streak but also to allow the progression and the transformation of the sickle's cells into a moving layer and to turn them into primitive streak-inducing cells, which probably does not occur without a leading hypoblastic edge. This explanation is also supported by some recent experiments of Khaner (personal communication) done with transplanted stage XIII sickles.

An interesting by-product of our study concerns the gradient of primitive streak inductivity in the hypoblast. According to our predictions based on the mode of formation of the hypoblast (Eyal-Giladi, 1991) and on recent labelling experiments (Eyal-Giladi et al., 1992) the posterior marginal zone cells start to enter into the posterior part of the forming

hypoblast and gradually spread in anterior and lateral directions. These cells do not, however, occupy the entire hypoblast, so that there are more of them in the posterior than in the anterior part of the hypoblast. The control experiments, in which we have applied only posterior or only anterior thirds of stage XIII hypoblast to epiblastic central discs, are very instructive (Table 1). Whereas with the posterior thirds, which probably contain relatively more cells of marginal zone origin, primitive streaks developed in 95% of the cases, with the complementary anterior thirds there was only 52% of primitive streak development in the central discs. In the control experiment (Table 3) with single hypoblastic fragments, which were cut arbitrarily from the hypoblast (so that some were probably posterior while others were anterior fragments), a primitive streak developed in 71.5% of the cases. Even more interesting is the comparison between the two subgroups in which only anterior hypoblastic thirds were used. The size of all the pieces of hypoblasts used was not exactly equal, because of the variability in the cutting of the hypoblasts. As a result, in some cases two anterior fragments were sufficient to cover the ventral surface of the epiblastic central disc, while in others three anterior hypoblastic fragments were needed. From the five cases in which two larger fragments were used, a primitive streak developed in 80% of the cases, whereas in the 14 cases in which three smaller fragments were used to cover the discs, a primitive streak developed in only 43% of the cases. In contrast, there are only minor differences between the subgroups of posterior fragments in which two or three fragments were applied to an epiblastic central disc. This fits the concept that the inductive cells of marginal zone origin are always in abundance in the posterior third whereas anterior thirds include less of them, especially when they were cut smaller. In the cases in which three anterior fragments had to be used to cover an epiblastic central disc, the number of inductive cells in each must have been relatively small, and in many cases not sufficient for the induction of a primitive streak.

Contrary to the doubts expressed by Izpisua-Belmonte et al. (1993) on whether the chick has a region corresponding to Nieuwkoop's center of the amphibian embryo, the induction of a primitive streak in the epiblast by the hypoblast can be compared to the induction of axial mesoderm (dorsal lip) in amphibians. It has been shown that an XTC cell-line conditioned medium can act as an inducer of mesoderm on both competent *Xenopus* ectoderm (Smith, 1987) and on chick epiblast (Mitrani and Shimoni, 1990). Also activin, when applied to a chick epiblast either via the culture medium or locally, can induce axial structures (Mitrani et al., 1990; Ziv et al., 1992). The above observations supported previous observations of Eyal-Giladi and Wolk (1970) who found that a hypoblast could induce a primitive streak in a competent epiblast even when the two were separated by a millipore filter, which led to the conclusion that a diffuseable inducing factor was secreted by the hypoblast, and was able to diffuse into the epiblast.

The clear differences between the inductivity of both the hypoblast and its supernatant on the one hand and the epiblast + marginal zone and their supernatants on the other hand as shown in the present experiments, led us to look also for a possible difference in the incubation media (= conditioned media) of hypoblast versus epiblast and marginal zone.

The electrophoretic analysis of the three blastodermic fragments (epiblast, hypoblast, area opaca+marginal zone) was done on quail because of the availability of the material at that time. However, we thought that the action of growth factors and embryonic inducers is such a basic and general event in development (similarity of action in amphibians and birds), that there could not be a major difference between the mesoderm inducing molecule in chick and quail. This is supported by the fact that chick and quail hypoblasts are interchangeable in induction studies. The electrophoretic study revealed a relative abundance of a 28×10^{-3} M_r protein in hypoblast cell lysates, and furthermore, very high concentrations of a protein with the same molecular mass in the hypoblast incubation medium. The abundance (approx. 50%) of this protein in the hypoblastic incubation medium and the fact that despite the much smaller total protein quantity in the incubation medium as compared to the hypoblast supernatant, the absolute amount of the 28×10^{-3} M_r protein is at least three times higher in the conditioned medium, suggests that the protein might be instrumental in the natural induction process.

Putting all the experimental data in this paper together, an interesting picture emerges. The hypoblast is the only fragment of a stage XIII blastoderm that is capable of inducing a primitive streak when directly applied to a competent epiblast. It was experimentally proved that it selectively synthesizes and secretes a 28×10^{-3} M_r protein. It therefore may be that the above protein is connected to primitive streak induction in the avian blastula.

Despite the fact that the inducing cells of the hypoblast were shown to be derived from the posterior marginal zone (Azar and Eyal-Giladi, 1979; Eyal-Giladi et al., 1992), we have shown unequivocally that the posterior marginal zone as such, cannot induce a primitive streak in a competent epiblastic central disc and that this inability cannot be accounted for by the difference in developmental age or by packaging of the marginal cell.

The posterior marginal zone cells become inductive only after they move via Koller's sickle into the primary hypoblast.

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