Development 125, 3521-3534 (1998)
Printed in Great Britain © The Company of Biologists Limited 1998
DEV4020

Induction of primitive streak and Hensen's node by the posterior marginal zone in the early chick embryo

Rosemary F. Bachvarova[1,*], Isaac Skromne[2] and Claudio D. Stern[2]

[1]Department of Cell Biology and Anatomy, Cornell University Medical College, 1300 York Avenue, New York, NY 10021, USA
[2]Department of Genetics and Development, College of Physicians and Surgeons of Columbia University, 630 West 168th Street, New York, NY 10032, USA
*Author for correspondence (e-mail: bachva@mail.med.cornell.edu)

Accepted 5 June; published on WWW 6 August 1998

SUMMARY

In the preprimitive streak chick embryo, the search for a region capable of inducing the organizer, equivalent to the Nieuwkoop Center of the amphibian embryo, has focused on Koller's sickle, the hypoblast and the posterior marginal zone. However, no clear evidence for induction of an organizer without contribution from the inducing tissue has been provided for any of these structures.

We have used DiI/DiO labeling to establish the fate of midline cells in and around Koller's sickle in the normal embryo. In the epiblast, the boundary between cells that contribute to the streak and those that do not lies at the posterior edge of Koller's sickle, except at stage X when it lies slightly more posteriorly in the epiblast. Hypoblast and endoblast (a second lower layer formed under the streak) have distinct origins in the lower layer, and *goosecoid* expression distinguishes between them.

We then used anterior halves of chick prestreak embryos as recipients for grafts of quail posterior marginal zone; quail cells can be identified subsequently with a quail-specific antibody. Anterior halves alone usually formed a streak, most often from the posterior edge. Quail posterior marginal zones without Koller's sickle were grafted to the anterior side of anterior halves. These grafts were able to increase significantly the frequency of streaks arising from the anterior pole of stage X-XI anterior halves without contributing to the streak or node. Stage XII anterior halves no longer responded. A *goosecoid*-expressing hypoblast did not form under the induced streak, indicating that it is not required for streak formation.

We conclude that the marginal zone posterior to Koller's sickle can induce a streak and node, without contributing cells to the induced streak.

Key words: Chick, Embryo, Induction, Primitive streak

INTRODUCTION

Among the most significant events in early development are those that confer organizer properties on one region of the embryo. The Spemann organizer of the amphibian embryo forms key elements of the dorsal axis and induces adjacent cells to form additional structures along the dorsal midline (Spemann and Mangold, 1924). In amphibians, the position of the organizer in the dorsal marginal zone is determined by the dorsal-ventral polarity established during cortical rotation following fertilization (Gerhart et al., 1986). In the blastula, the dorsal-vegetal region is able to induce an organizer in animal cap cells (Nieuwkoop, 1969; Boterenbrood and Nieuwkoop, 1973; Nieuwkoop, 1977). Moreover, transplantation of the dorsal-vegetal cells of the 64-cell embryo can rescue the axis of a UV-irradiated embryo or produce a second axis in a normal embryo (Gimlich and Gerhart, 1984; Gimlich, 1986). These experiments have defined the amphibian Nieuwkoop Center as a dorsal-vegetal region capable of inducing an organizer in adjacent animal cells, without contributing to the induced axial structures. The basis of Nieuwkoop Center activity is believed to be release of one or more TGFβ factors such as Vg1 or activin (see Slack, 1994), and activation of the pathway downstream of Wnt signaling (see Harland and Gerhart, 1997). Transplantation of future organizer cells from the dorsal equatorial region to a UV-treated embryo can restore axis formation, but the transplant contributes directly to the new axis (Gimlich, 1986). In this case the new axis is said to be organized by the transplant, i.e. it is formed by self-differentiation of transplanted cells and by recruitment of host cells to dorsal axial structures. Recently, it has become apparent that in the normal amphibian embryo, formation of the organizer may involve autonomous processes within future organizer cells, including release of some of the same factors produced by the Nieuwkoop Center (see Harland and Gerhart, 1997). Thus, it may be said that the future organizer has Nieuwkoop Center activity; nevertheless, the Nieuwkoop Center as originally described is a dorsal-vegetal region that can induce an organizer in adjacent animal cap cells without contributing to it.

Various attempts have been made to define a region of the avian embryo that is functionally equivalent to the Nieuwkoop Center. If the avian embryo possesses such a region, it should be capable of inducing an organizer (Hensen's node) in an

adjacent region, and cells from the inducing region should not contribute to the organizer. The search has focused on (1) Koller's sickle (hereafter referred to as the sickle), (2) the hypoblast and (3) the posterior marginal zone (MZ) (see Stern, 1990, and the fate-mapping section below for diagrams of the early chick embryo).

Grafting of the sickle to a new site can initiate an ectopic streak (Izpisua-Belmonte et al., 1993; Callebaut and van Nueten, 1994; Callebaut et al., 1997). However, sickle and associated middle layer cells contribute to the node in normal development (Izpisua-Belmonte et al., 1993), and these grafts probably contain future organizer cells.

Some evidence suggests that the hypoblast of the prestreak embryo is capable of inducing a streak in a new position when rotated by 90°-180° (Waddington, 1932, 1933; Azar and Eyal-Giladi, 1981). However, the origin of the streak is difficult to assess, due to the tendency of the rotated hypoblast to bend the original streak (Waddington, 1932, 1933). Also, it is possible that the ectopic streak appears by migration of predetermined cells toward a new position dictated by the hypoblast rather than by a change in fate of adjacent cells, or that middle layer cells contributing to the organizer may have been moved with the hypoblast. Moreover, recent attempts to demonstrate initiation of an ectopic streak by rotating the hypoblast were unsuccessful (Khaner, 1995). Nevertheless, the hypoblast expresses factors suggesting it has a positive role in streak formation, including activin (Mitrani et al., 1990) and Vg1 (Seleiro et al., 1996), as well as several transcription factors implicated in streak formation in later development, such as *gsc* (Hume and Dodd, 1993 and see below), *otx2* (Bally-Cuif et al., 1995), and *Hnf3*β (Ruiz-i-Altaba et al., 1995). However, there is no direct evidence that the hypoblast can induce cells to take on new fates.

In considering the role of the hypoblast, it is important to clarify the nature of the successive lower layers that are present before the embryonic endoderm appears from cells migrating from the streak. It is generally agreed that the hypoblast forms a complete lower layer under the central disc epiblast by stage XIII (prestreak embryo stages, according to Eyal-Giladi and Kochav, 1976). It is often assumed that the hypoblast remains in this position until replaced by the endoderm coming from the primitive streak. However, this does not account for the fact that most of the hypoblast is found in the anterior germinal crescent by the primitive streak stage 3+ to 4 (stages according to Hamburger and Hamilton, 1951). It has been suggested that the lower layer appearing under the central disc as the streak forms at stage 2-3 represents a new layer distinct from the hypoblast (Vakaet, 1970). In this view, as the streak forms and lengthens, the hypoblast is displaced toward the germinal crescent anterior to the streak, and a new lower layer expands from the posterior under the central disc. Both Spratt and Haas (1960a) and Vakaet (1970) describe a change in the movements of particles placed on the lower layer as the streak forms; rather than a sheet extending anteriorly, the sickle itself appears to undergo expansion in all directions from within. This new lower layer can be distinguished from the original hypoblast cells by its smaller cells (Stern and Ireland, 1981). It was referred to as 'sickle endoblast' by Vakaet (1970) but, to avoid presumptions about its origin, we will refer to it as 'endoblast'. Most of the experiments on rotation of the 'hypoblast' have not distinguished between hypoblast and endoblast.

Rotation or transplantation of the posterior MZ of prestreak embryos by 90° can result in formation of a streak in the new position (Khaner and Eyal-Giladi, 1986, 1989; Eyal-Giladi and Khaner, 1989; Khaner, 1998). However, in these experiments, cells predetermined to form streak may migrate to the new site of the posterior MZ. Moreover, part or all of the sickle and overlying epiblast were included in the posterior MZ piece. Future organizer cells are present in the epiblast very near the sickle at early prestreak stages (Hatada and Stern, 1994), and in the middle layer of cells associated with the sickle in prestreak stages (Izpisua-Belmonte et al., 1993). Thus, it is likely that the transplanted piece included future organizer cells. Eyal-Giladi and colleagues have proposed that the inductive activity of the posterior MZ is not direct; rather posterior MZ only becomes inductive after it migrates ventrally into the sickle and then anteriorly to form an inductive hypoblast (Azar and Eyal-Giladi, 1979; Eyal-Giladi et al., 1992, 1994).

In the initial experiments reported here we have sought to confirm that a second lower layer distinct from the hypoblast is formed under the central disc as the primitive streak forms. We have identified *goosecoid* (*gsc*) as a molecular marker that is expressed in the hypoblast and not in the endoblast, and thus can be used to distinguish between the two.

We have also undertaken accurate fate-mapping of the posterior midline region in order to establish the boundaries of node and primitive streak precursors. We find that the primitive streak receives contributions from the epiblast and middle layer just anterior to and within the sickle, but that the MZ posterior to the sickle makes only a small, if any, contribution to the streak. This posterior MZ region was then used in the transplant experiments. In addition, fate-mapping showed that the hypoblast and endoblast originate from different locations in the deep layers of the sickle region, providing a further means of distinguishing between these two layers.

In the main set of experiments, we investigated whether the posterior MZ represents a functional equivalent of the Nieuwkoop Center, able to induce a streak without contributing to it. To test for induction, we grafted quail posterior MZ to the anterior side of chick anterior halves, and scored the appearance of a chick streak and node oriented from the graft (at 180° from the original predicted position of the streak) as indicating induction of a streak. The normal fate of the anterior half at stage X-XI is to produce neural tissue, surface ectoderm, extraembryonic ectoderm and a small amount of lateral mesoderm (Hatada and Stern, 1994). We show that grafting quail posterior MZ (without the sickle) to the anterior side of chick anterior halves results in the appearance of organizer (node) and primitive streak in anterior cells without a contribution of donor cells to these structures. DiI/DiO-labeling experiments indicate that the formation of the ectopic streak involves a change in cell fate. By stage XII, anterior halves are no longer able to respond. A *gsc*-expressing hypoblast does not form under the induced streak; rather endoblast forms, primarily from chick cells. We conclude that the posterior MZ can induce a streak and node without contributing to these structures.

MATERIALS AND METHODS

Fate mapping, photography and histology

Unincubated, or briefly incubated, fertile White Leghorn hens' eggs

(SPAFAS, CT, USA) were set up for modified New culture (New, 1955; Stern and Ireland, 1981). Embryos were staged according to Eyal-Giladi and Kochav (1976), freed from the vitelline membrane and carefully cleaned with gentle streams of buffer to reveal Koller's sickle without removing the delicate deep layers of the MZ. All manipulations were performed in Pannett-Compton saline (PC; Pannett and Compton, 1924).

Different procedures were used to label cells in different layers. To label dorsal cells, embryos were turned over to expose the epiblast layer and labeled with either the carbocyanine dye DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethyl indocarbocyanine perchlorate, Molecular Probes Inc.) or with DiO (3,3′-dioctadecyl oxacarbocyanine perchlorate, Molecular Probes Inc.) as described previously (Stern, 1990). A small volume of dye solution was applied to one of four different positions in the mid-line of the embryo using gentle air pressure. Application of DiO to a second position was used as a reference point. Using this method, 20-40 cells are usually labeled. From 125 labeled embryos, 61 that provided clearcut information were considered in our analysis.

Labeling of ventral and middle layer cells was done from the ventral side, in one of six positions. DiI was applied iontophoretically using Al-Si glass micropipettes with an internal filament (A-M Systems, Inc. WA, USA) pulled using a Kopf vertical electrode puller. A watch glass containing the vitelline membrane with the embryo was placed on the dissecting microscope stage. PC solution was added around the vitelline membrane and a small amount of PC solution was left covering the embryo. A copper wire connected to the (–) end of a 9 V battery was submerged in the buffer outside the vitelline membrane. Another copper wire was inserted into a micropipette filled with 0.5% DiI in absolute ethanol and the other end of the wire was connected to the (+) end of the battery. The tip of the micropipette was brought to the appropriate cell layer using a micromanipulator and a 1-2 minute pulse of current applied. At the end of the pulse, the polarity was reversed and a 5 second pulse applied to clear the dye from the tip of the micropipette. This step minimizes non-specific labeling due to dye precipitation that sometimes occurs at the tip of the micropipette, which can deposit itself on the embryo when the micropipette is removed. Groups of 2-10 cells are labeled using this method. However, since the extensive cell movements that occur at these developmental stages make it impossible to follow small groups of cells, labeling of 8-10 cells was preferred. From 107 embryos labeled by this method, 84 were considered for analysis.

After labeling, the embryos (ventral side up) and their supporting vitelline membranes were transferred to 3 cm Petri dishes containing egg albumin. The posterior end of the embryo was labeled with carbon particles (lampblack carbon, Fisher) for reference. Labeled embryos were examined by epifluorescence microscopy in a Vanox-T microscope. Photographs were taken at time 0 and every 6-12 hours during the incubation period using epifluorescence and bright-field optics on Fuji 1600 ASA film. Incubation was carried out in a humidified chamber at 38°C for up to 60 hours and embryos were staged according to Hamburger and Hamilton (1951).

To confirm the site of labeling, control embryos were fixed at time 0 in 4% formaldehyde in PBS. DiI/DiO fluorescence was photo-converted by exposure to the excitation wavelength in the presence of 50 μg/ml 3,3′-diaminobenzidine (DAB, Sigma) in 0.1 M Tris, pH 7.4 (Stern, 1990). Embryos were dehydrated, embedded in Paraplast, and sectioned. In 17/22 cases (77%), only the desired position and layer had been labeled.

Grafts

Unincubated fertile White Leghorn hens' eggs (SPAFAS, CT) or quails' eggs (Karasoulas, CA) were opened and the yolks floated in PC solution. Vitelline membranes were set up for New culture (New, 1955), usually on watch glasses, or in a few cases in 3 cm Petri dishes. Embryos were placed in Tyrode's solution, the yolk plug removed with care leaving some deep MZ attached to the epiblast, staged according to Eyal-Giladi and Kochav (1976), and those with a clear polarity used for the experiments. Incubation of 12 stage X-XIII whole embryos with the posterior pole marked indicated that the average error in estimation of the anterior-posterior axis was 15°. Chick embryos were cut into anterior and posterior halves with a glass needle laid down horizontally. The anterior half was placed on a vitelline membrane ventral side up. The posterior region of a quail embryo (including MZ and area opaca) was cut off. This posterior piece was cut in one of three positions: including the sickle, through the sickle, or posterior to the sickle. Usually the cut was curved to conform with the curve of the sickle. The size of the piece was approximately 2.5×0.5 mm. This piece was moved either to the posterior or anterior side of a chick anterior half. For transplants to the anterior side, an anterior piece of similar size, including full or almost full width of the MZ, was removed from the chick embryo and placed adjacent to the posterior edge of the anterior half. Tyrode's solution was removed by aspiration with a fine pipette and the pieces brought together with steel needles. A small amount of carmine powder was placed in the middle of the area opaca in the quail piece. Controls consisted of chick anterior halves with an anterior piece of a quail embryo applied to the posterior edge. The transplants were incubated in New culture (New, 1955) at 38°C in a humid environment; typically they were observed at 18 hours and fixed in 4% paraformaldehyde in PBS plus 2 mM EGTA at 22 hours or whenever a definitive streak (stage 3+ to 4) had appeared. Embryos were stored in methanol at –20°C.

Whole-mount in situ hybridization and immunohistochemistry

Whole-mount in situ hybridization on the transplants was performed essentially as described (Streit et al., 1997), using digoxigenin-labeled riboprobes for *Brachyury* (*Ch-T*) (the *Ch-T* plasmid was a kind gift of Dr J. Smith), *gsc* (Izpisua-Belmonte et al., 1993) and *chordin* (Streit et al., 1998). In most cases, proteinase K treatment was omitted and hybridization proceeded for 7 hours at 65°C for *Ch-T* and 68°C for *gsc* and *chordin*. After the color reaction with NBT/BCIP, embryos were rinsed, fixed in paraformaldehyde for 1 hour, and whole-mount immunohistochemistry using the QCPN antibody directed against quail antigen on embryos with or without previous in situ hybridization was carried out as described (Streit et al., 1997), using anti-mouse IgG-HRP as the secondary antibody. The monoclonal mouse IgG QCPN was developed by Dr B. M. Carlson and obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, The Johns Hopkins University School of Medicine, Baltimore MD 21205 and the Department of Biological Sciences, University of Iowa, Iowa City, Iowa 52242, under contract N01-HD-2-3144 from the NIHCD. Embryos were photographed, and some were dehydrated through methanol, propanol and tetrahydronaphthalene, embedded in Paraplast and sectioned at 10 μm.

Terminology for structures of the prestreak embryo

The stage X (prestreak stages are designated by Roman numerals according to Eyal-Giladi and Kochav, 1976) chick embryo at the time of laying is a disc consisting of epiblast, a simple epithelium, underlain by a partially formed lower ventral layer (see Fig. 2 and Stern, 1990). The epiblast is subdivided into the central disc, a surrounding ring called the marginal zone (MZ), and the outermost ring, the area opaca.

The lower layer of the area opaca (germ wall) consists of large yolky cells adjoining the yolk below. The MZ epiblast is underlain by the deep layer of the MZ, or the germ wall margin. This layer may be several cells thick, is quite loosely attached, and includes a projection ventral to Koller's sickle (labeled 9 in Fig. 2). The sickle consists of an approximately 90° arc of more densely packed lower layer cells that defines the boundary of the central disc and MZ epiblast. Within the central disc, the lower layer consists of a few scattered groups of

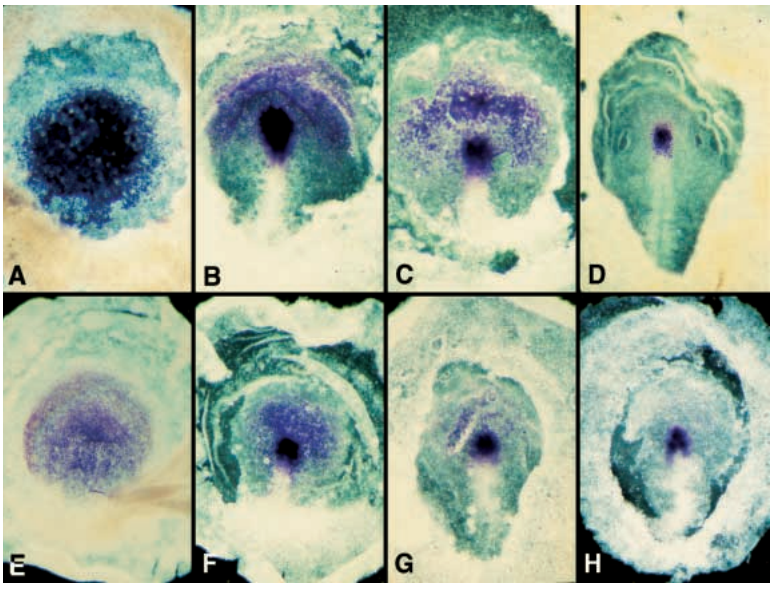
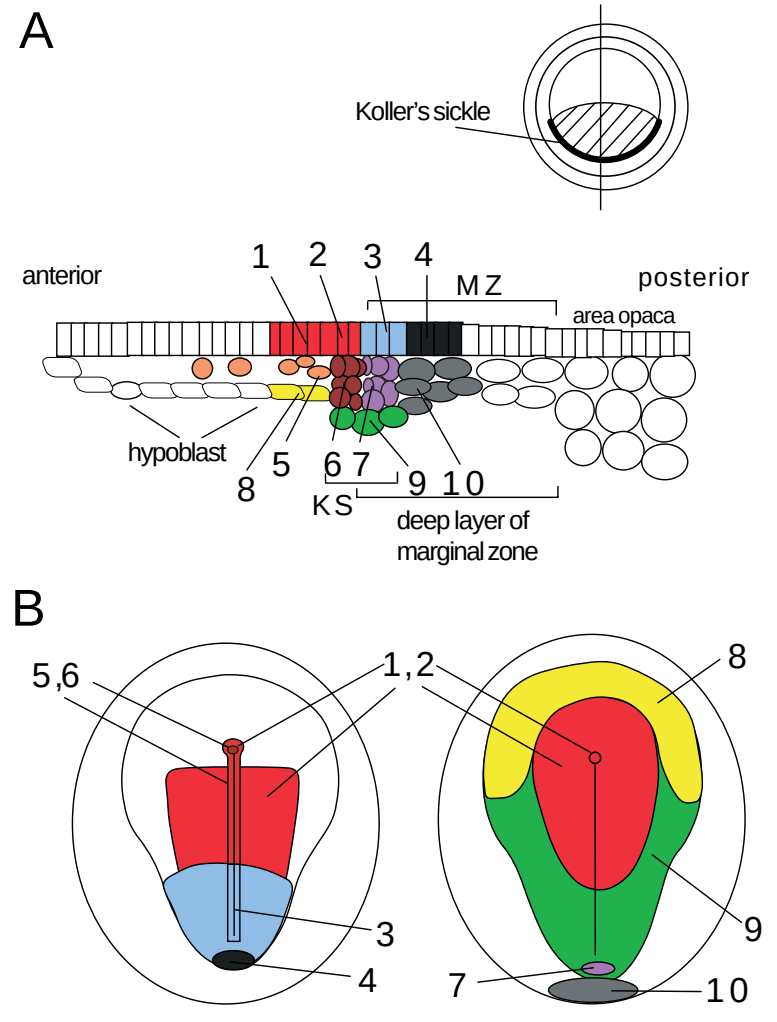


Fig. 1. Expression of *gsc* in whole mounts of chick and quail embryos. (A-D) chick, (E-H) quail. (A,E) Stage XIII showing the *gsc*-expressing hypoblast. (B-C, F-G) Stage 3, showing the progressive reduction of the *gsc*-expressing region in the lower layer, while the node expresses *gsc*. (D,H) Stage 4–. Expression is restricted to the node.



cells or 'islands'. The islands are generally believed to arise from the central disc epiblast by polyingression (Peter, 1938). Between stages X and XIII, the lower layer is completed by cells migrating anteriorly from the posterior region; these merge with the islands to form a complete hypoblast under the central disc by stage XIII (Vakaet, 1970; Eyal-Giladi and Kochav, 1976; Stern and Ireland, 1981; Stern, 1990). The hypoblast moves to the germinal crescent by stage 4 (Vakaet, 1970).

A few middle layer cells are usually found between the hypoblast and the epiblast anterior to the sickle (Izpisua-Belmonte et al., 1993). The primitive streak appears at stage XIV-2, and a distinct node forms by stage 3+ (primitive streak stages in Arabic numbers according to Hamburger and Hamilton, 1951). In the lower layer, the endoblast apparently forms during stages 2-3, spreading out from the region of the sickle as the hypoblast recedes toward the anterior germinal crescent.

RESULTS

Goosecoid expression distinguishes the hypoblast from the endoblast

At stage XIII, the newly completed hypoblast expresses *gsc* throughout (Fig. 1A). At stage 2, the most posterior part of the lower layer no longer expresses *gsc* (not shown), and during stage 3, the non-expressing region expands to include the region beside the streak (Fig. 1B,C). By stage 4, the lower layer no longer expresses *gsc*, including the hypoblast in the germinal crescent (Fig. 1D). In quail embryos, a similar expression pattern is seen (Fig. 1E-H). The expression of *gsc* in the hypoblast is more obvious in the White Leghorn strain than in the Rhode Island Red or HiSex Brown strains (cf. Hume and Dodd, 1993; Izpisua-Belmonte et al., 1993; C. D. Stern, unpublished results).

Fig. 2. (A) Diagram of a midsagittal section of a prestreak embryo at about stage XII showing the various structures and the sites of labeling with DiI/DiO. The diagram in the upper right indicates the plane of section on a ventral view of a stage XII embryo; the hypoblast is hatched. Sites of injection: (1) epiblast anterior to the sickle; (2) epiblast over the anterior portion of the sickle; (3) epiblast over the posterior portion of the sickle; (4) epiblast of the MZ just posterior to the sickle; (5) middle layer cells just anterior to the sickle; (6) cells within the anterior portion of the sickle; (7) cells within the posterior portion of the sickle; (8) hypoblast just anterior to the sickle; (9) the portion of the deep layer of the MZ, which extends ventral to the sickle; (10) deep layer of the posterior MZ just posterior to the sickle. KS, Koller's sickle; MZ, marginal zone. (B) Diagrams of a stage 4 embryo showing the positions of cells derived from the labeled sites. For details, see Table 1. (Left diagram) Epiblast and mesoderm layers. Sites 1,2,5 and 6 contribute to the node, anterior streak and embryonic mesoderm (red/brown). Site 3 contributes to posterior primitive streak and extraembryonic mesoderm (blue). Site 4 of a stage X embryo labels the posterior primitive streak (not shown), and site 4 of stage XI-XIII embryos remains in the posterior area opaca (dark grey). (Right diagram) Lower layers. Sites 1 and 2 contribute to embryonic endoderm (red). Site 8 is found in hypoblast in the germinal crescent (yellow), site 9 is found in endoblast (green). Sites 7 and 10 remain posterior to the streak (pink and light grey, respectively).

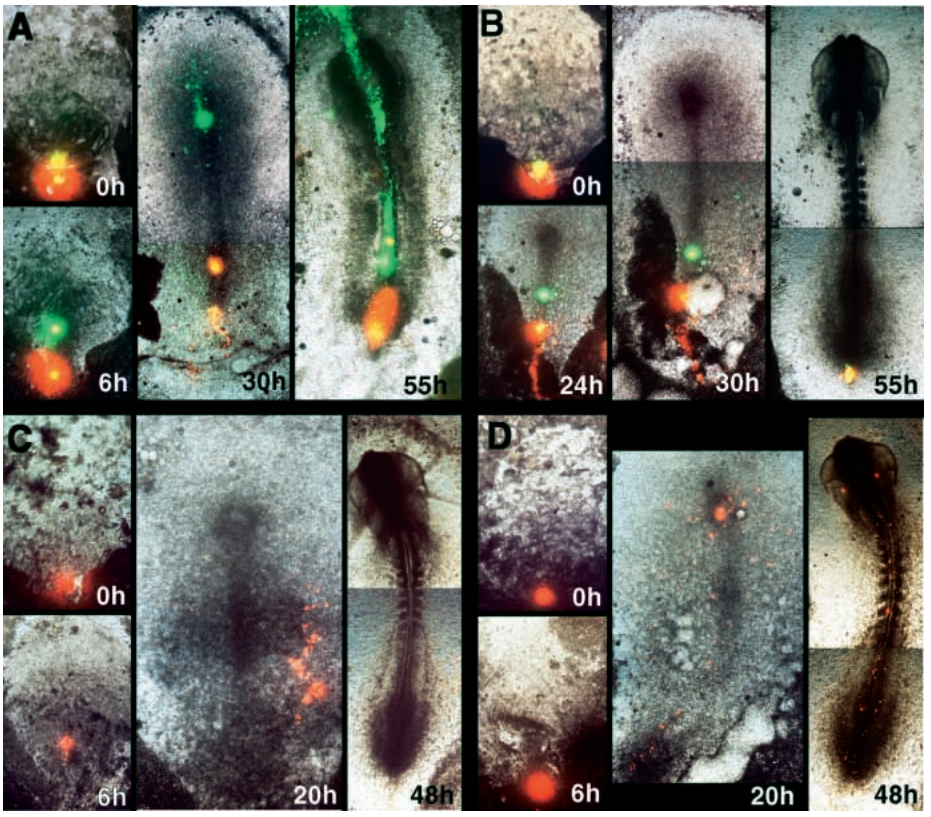


Fig. 3. Examples of embryos labeled at the posterior midline at prestreak stages and followed for the indicated hours of incubation. Embryos reach stage 4 by about 24 hours of incubation and somite stages by 48-55 hours. (A) Pressure injection of DiO (green) into the epiblast of a stage XII embryo dorsal to the anterior sickle (site 2) and of DiI (red) into the epiblast dorsal to the posterior sickle (site 3). At 30 hours, the labels are seen primarily in the node and posterior streak, respectively. (B) Pressure injection of DiO into the epiblast dorsal to the posterior sickle of a stage XIII embryo (site 3) and with DiI into the posterior MZ just posterior to the sickle (site 4). (C) Iontophoretic DiI labeling of a stage XI embryo in the deep layer of the posterior MZ ventral to the sickle (site 9). At 20 hours the label is found in the endoblast. (D) Iontophoretic DiI labeling of a stage XII embryo in the anterior sickle (site 6). At 20 hours, labeled cells are found primarily in the anterior primitive streak and migrating mesoderm.

At stages 2 and 3 the staining region corresponds closely to the position of the hypoblast and the non-staining region to the sickle endoblast, which was described by Vakaet (1970) as arising at the time of streak formation, displacing the hypoblast anteriorly. We therefore adopted *gsc* expression in the lower layer as a marker for hypoblast, distinguishing it from endoblast.

Fate map of the region in and around Koller's sickle

The aims of the fate-mapping experiments were: (1) to define accurately in the normal embryo the boundary between cells contributing to the streak and those remaining caudal to it and (2) to determine whether the endoblast has a different origin from that of the hypoblast.

Embryos were labeled with DiI/DiO in the midline at pre-primitive streak stages at the ten sites indicated in Fig. 2A. In all cases, labeled cells were found primarily in only one of the structures scored in stage 4 primitive streak embryos at about 24 hours of incubation. To confirm the results, embryos were also examined at somite stages (48-55 hours of incubation) and were observed to contain labeled cells in the structures predicted from the earlier labeling pattern. The results obtained at 24 hours are summarized in Table 1 and Fig. 2B, and examples presented in Fig. 3. The main conclusions are given below.

Epiblast anterior to the sickle or dorsal to the anterior part of the sickle contributed most often to the node and anterior part of the streak (Fig. 3A), with the contribution to the node decreasing in later stages. Epiblast cells dorsal to the posterior part of the sickle contributed most often to posterior streak (Fig. 3A,B). MZ epiblast posterior to the sickle usually contributed to posterior streak when labeled at stage X, and to posterior area opaca when labeled at stages XI-XIII (Fig. 3B). Thus, in the epiblast, the boundary between cells that do and do not contribute to the streak lies at the posterior edge of the sickle from stage XI.

Middle layer cells just anterior to the sickle as well as anterior sickle itself contributed to the node and anterior primitive streak (Fig. 3D), while posterior sickle contributed to posterior streak. These results are consistent with earlier findings (Izpisua-Belmonte et al., 1993).

Labeling of the lower layer gave the following results. At stages X-XIII, hypoblast just anterior to the sickle contributed to hypoblast found in the germinal crescent. The portion of the deep layer of the MZ lying ventral to the sickle formed endoblast (Fig. 3C). Deep MZ just posterior to the sickle remained caudal to the primitive streak in the area opaca. Therefore, the hypoblast and endoblast have distinct origins in the lower layers.

Choice of anterior halves to test for the inducing activity of posterior MZ

Results in the literature suggested that it is relatively difficult to initiate an ectopic streak using grafts to whole pre-streak embryos (Spratt and Haas, 1960b; Khaner and Eyal-Giladi, 1989; Eyal-Giladi and Khaner, 1989). In a pilot study, the ability of a piece of posterior MZ/area opaca to initiate a streak at a new site was tested by grafting to the anterior side of whole stage X-XI embryos. Posterior MZ was cut to include or exclude the sickle (see Fig. 4). In 7/7 cases in which the posterior piece lacked the sickle, only a host streak oriented from the posterior pole appeared; in 3/3 cases in which the

transplanted piece included all or most of the sickle, a second streak appeared oriented from the anterior pole. Thus, grafts of posterior MZ without the sickle and without cells that contribute to the streak are unable to induce a streak in a whole embryo.

We next cultured isolated anterior halves of embryos to determine whether they were suitable to use as hosts for grafts of quail posterior MZ. For these cultures, a piece of quail anterior MZ and area opaca was grafted to the posterior cut edge of the chick anterior half (Fig. 4, top diagrams), to prevent conversion of the cut edge of the central disc to area opaca-like cells (Spratt and Haas, 1960b). After 18-24 hours of incubation, 43% of these cultures developed a streak oriented from the posterior, 30% from lateral positions, and only 9% from the anterior pole (17% did not form a streak) (controls in Table 2A and Fig. 6A). The low frequency of streaks from the anterior pole indicates that these anterior halves may be used to test the effects of a grafted posterior MZ.

Table 1. Fates of cells labeled in different regions of the embryo

Labeling site			Labeled structures at stage 4							
Layer		Stage	Hensen's node (HN)	Anterior primitive streak (APS)	Posterior primitive streak (PPS)	Germinal crescent (GC)	Endoblast (End.)	Posterior Area Opaca (AO)	*n*	Predominant contribution
Upper layer	1. Epiblast anterior to sickle	X	(100)						1	HN
		XI	80		20				5	HN
		XII	66	33					6	HN
		XIII	44	44	12				9	HN+APS
	2. Epiblast dorsal to anterior sickle	X	(100)						1	HN
		XI	34	50				16	6	APS
		XII	15	57	28				7	APS
		XIII		70	30				10	APS
	3. Epiblast dorsal to posterior sickle	X			84			16	6	PPS
		XI		16	84				6	PPS
		XII		16	84				6	PPS
		XIII	5	22	64			9	22	PPS
	4. Epiblast posterior to sickle, posterior MZ	X			80			20	5	PPS
		XI			34			66	6	AO
		XII	17		17			66	6	AO
		XIII		10	25			65	20	AO
Lower layers	5. Middle layer cells	X	(100)						1	HN
		XI	(100)						1	HN
		XII		100					2	APS
		XIII		100					2	APS
	6. Anterior sickle	X	50	50					2	HN+APS
		XI	33	67					6	APS
		XII		100					7	APS
		XIII		100					8	APS
	7. Posterior sickle	X								
		XI			(100)				1	PPS
		XII			(100)				1	PPS
		XIII			50			50	4	PPS+AO
	8. Posterior hypoblast	X								
		XI				(100)			1	GC
		XII				(100)			1	GC
		XIII				100			3	GC
	9. Deep layer of posterior marginal zone ventral to sickle	X					100		3	End.
		XI					100		3	End.
		XII					100		5	End.
		XIII		16			84		6	End.
	10. Deep layer of marginal zone just posterior to sickle	X						100	2	AO
		XI						100	2	AO
		XII						100	6	AO
		XIII			6			94	17	AO

Small groups of cells located at various sites in embryos at stages X-XIII were labeled with DiI or DiO as described in Materials and methods. The numbers for the sites of injection correspond to the numbered labels in Fig. 2A. The embryos were allowed to develop overnight to approximately stage 4 and the structure containing most of the labeled cells scored. The percentage of the embryos in each sample with cells predominantly in a given structure is presented.

The abbreviations in the last column refer to the structure receiving the predominant contribution of labeled cells from a given injection site, and correspond to the headings of the columns in the body of the table.

n, number of embryos in the sample.

The numbers in parentheses indicate cases in which sample size was 1.

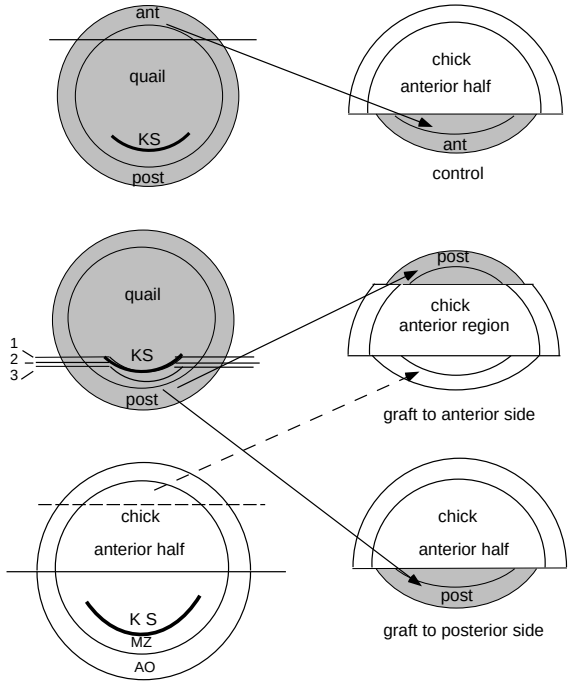


Fig. 4. Design of grafts of regions of quail embryos to chick anterior halves. Chick embryos were cut into anterior and posterior halves, and the anterior half was placed on a vitelline membrane. For controls, a quail anterior piece was positioned next to the posterior edge of a chick anterior half. For experimental samples, quail embryos were cut (1) just anterior to the sickle, (2) through the sickle or (3) just posterior to the sickle. For grafts to the anterior side, an anterior piece was cut off the chick anterior half and moved to the posterior edge of the anterior half; the quail posterior piece was then positioned next to the anterior edge of the chick anterior piece. For grafts to the posterior side, the quail posterior region was positioned adjacent to the posterior edge of the chick anterior half. In some cases, the quail posterior piece was positioned at the anterior side of a whole chick embryo (not shown).

Posterior MZ grafted to the anterior side of anterior halves positions the primitive streak

To test the ability of posterior MZ to determine the position and orientation of the primitive streak, quail stage X-XII posterior MZ was grafted to the anterior side of chick stage X-XI anterior halves. The posterior piece in all cases included the posterior MZ and the more peripheral posterior area opaca (Fig. 4). The latter has little effect on development (Spratt and Haas, 1960b), providing primarily tension (Bellairs et al., 1967), and is not considered hereafter. In the posterior MZ graft, two layers were present: the epiblast, and an underlying deep layer which was sometimes continuous and sometimes a discontinuous network of cells (see Fig. 5A). As shown in Fig. 4, the grafts were cut just anterior to the sickle, through the sickle, or just posterior to the sickle, which was clearly visible in the quail embryos selected as donors (Fig. 5A,B). As in the controls, the cut edge of the anterior half was sealed with a piece of MZ plus anterior area opaca (Figs 4, 5C). After 18-24 hours of incubation, embryos were examined for the presence and orientation of a primitive streak.

When the sickle was not included in the graft, a streak was formed originating from the anterior pole in 43% of the cases (Table 2A, Fig. 6C), significantly more than the 9% observed in controls (Fig. 6A) ($P \leq 0.005$, χ^2 test). When the sickle was included in the graft, a streak was formed from the anterior pole in 88% of the cases (Table 2A, Fig. 6E). When the graft was cut through the sickle, an intermediate value was obtained (Table 2A).

Table 2. Positioning of the primitive streak in anterior halves of chick embryos by the posterior marginal zone of quail embryos

A. Stage X-XI chick anterior halves

		Streak oriented from			No streak
	n	Posterior %	Lateral %	Anterior %	%
Control anterior halves[a]	23	43	30	9	17
Grafts to the anterior side					
Donor posterior piece					
No sickle[b]	51	10	14	43*	33
Through sickle	9	0	11	67	22
With sickle[c]	16	0	0	88	13
Grafts to the posterior side					
Donor posterior piece					
No sickle[d]	19	95**	5	0	0
Through sickle	4	100	0	0	0
With sickle	20	95	0	0	5

B. Stage XII-XIII chick anterior halves

		Streak oriented from			No streak
	n	Posterior %	Lateral %	Anterior %	%
Control anterior halves	27	48	22	0	30
Grafts to the anterior side					
Donor posterior piece					
No sickle	19	42	21	5	32

*Development of a streak from the anterior side is significantly correlated with grafting of a posterior piece to the anterior side; $P \leq 0.005$, χ^2 test.
**Development of a streak from the posterior is significantly correlated with grafting of a posterior piece to the posterior side; $P \leq 0.001$, χ^2 test.
[a]Shown in Fig. 6A; [b]Fig. 6B; [c]Fig. 6C; [d]Fig. 6D.

Most of the primitive streaks appeared morphologically normal, while 15% appeared abnormal with an enlarged node, protruding ventrally, and a short streak. When analyzed by in situ hybridization for the expression of the streak marker *Ch-T* (7 embryos) or the node markers *gsc* (8 embryos) or *chordin* (Streit et al., 1998) (4 embryos), all samples revealed appropriate staining (Figs 7A,D,I, 8D,F).

Of those grafted embryos that did not form a streak, some formed an area of more densely packed cells, but did not go on to form a streak; in others, the central disc became relatively thin and clear with little lower layer. The fraction of grafted embryos not forming a streak was 13% in those with a graft including the sickle, and 17% in controls. It was highest, 33%, in those receiving a graft to the anterior side without the sickle; this effect may stem primarily from the regulatory processes occurring within the anterior half (see Discussion), rather than from technical problems.

We conclude that the posterior MZ (without sickle) is able to determine the position of the streak. The induced node expresses *gsc* and *chordin*, and the streak expresses *Ch-T*.

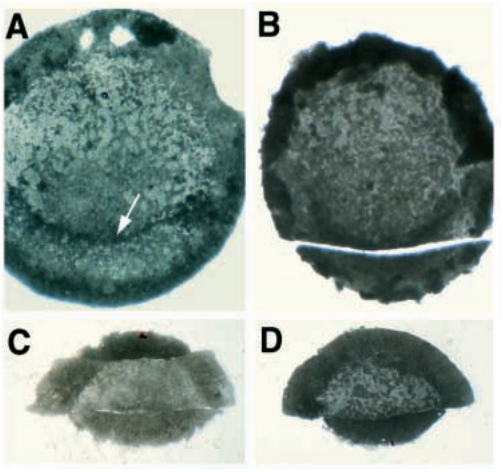


Fig. 5. Prestreak embryos and grafted embryos at start of incubation. Posterior is down in all panels. (A) A stage XI+ chick embryo. Note the sickle (arrow) and the layer of deep MZ cells posterior to it. (B) A stage XII quail embryo cut just posterior to the sickle. (C) Graft of posterior MZ to the anterior side of an anterior half of a chick embryo. (D) Graft of posterior MZ to the posterior side of an anterior half of a chick embryo. Bright field illumination.

Distribution of quail cells after grafting to the anterior side of chick anterior halves

(1) Grafts of posterior MZ without the sickle do not contribute cells to the streak or node

The contribution of quail cells from the graft to the ectopic streaks was assessed using immunohistochemical staining for the quail perinuclear antigen QCPN. After grafting posterior MZ without the sickle, quail cells were not found in the epiblast, streak, or node. Quail cells were found in a narrow

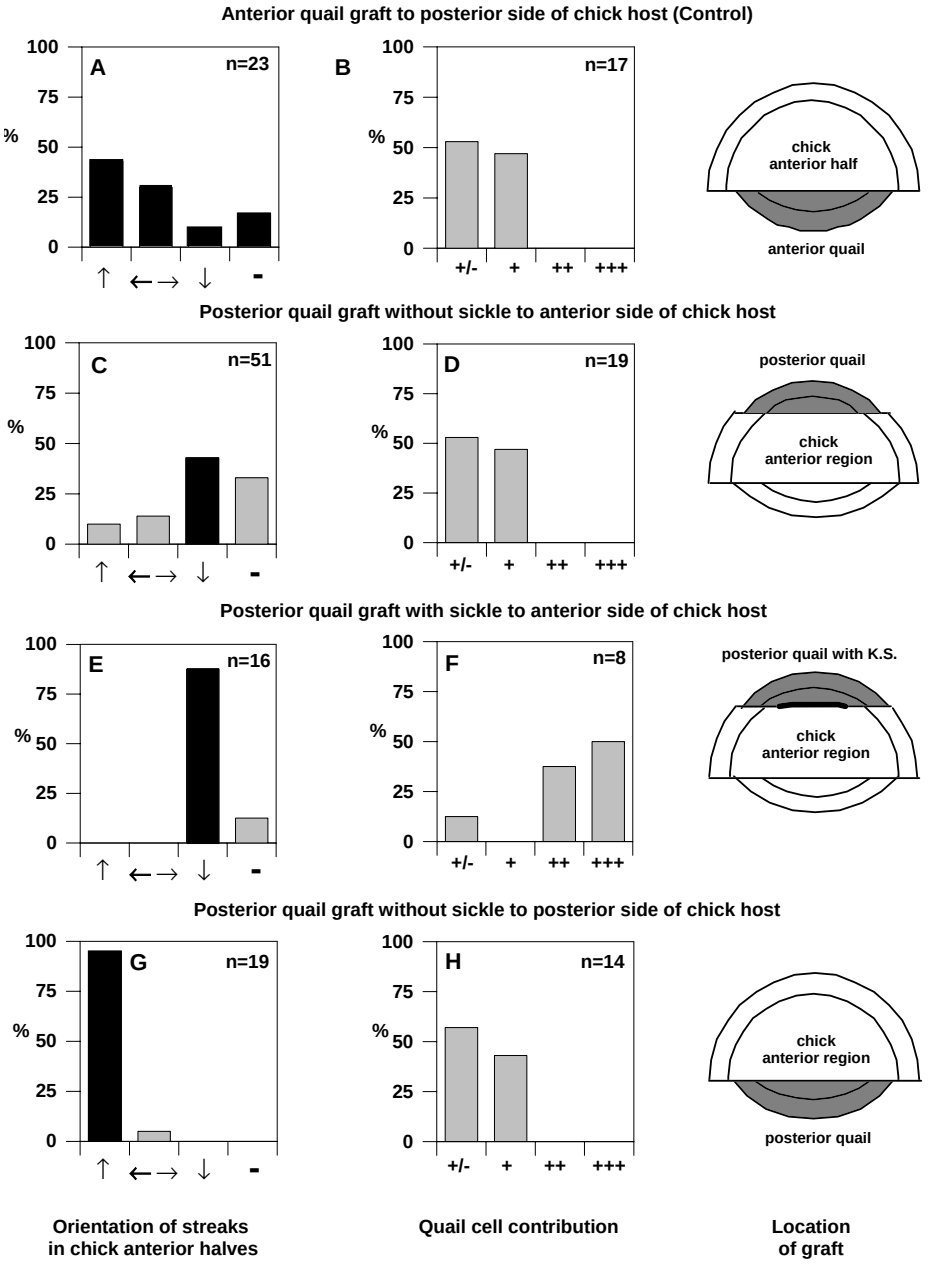


Fig. 6. Frequency of appearance of primitive streaks in different orientations and the contribution of quail cells after grafting quail MZ to anterior halves of chick embryos. Different types of quail grafts of MZ were carried out as shown in the diagrams on the right. (A,C,E,G) Percentage of grafted embryos forming a streak oriented as indicated by the arrows. ↑, oriented from the posterior; ← →, from a right or left lateral position; ↓, from the anterior; –, no streak formed. *n*, total number in the sample. Darker bars in C,E,G indicate streaks oriented from the graft. The data are included in Table 2A. (B,D,F,H) Percentage of embryos with different quantities of quail cells in the region of the primitive streak. In B, all samples are included. In D,F,H only the results for samples with a streak oriented from the graft are presented. +/–, 0-10 quail cells in the lower layer under the primitive streak region. +, up to half the cells in the lower layer were quail cells; ++, most or all of the lower layer cells were quail cells; +++, same but with an additional small contribution of quail cells to the midline epiblast of the primitive streak. *n* = total number in sample.

band in the lower layer extending centrally a short distance from the graft but not reaching the streak (Fig. 7A). A few individual quail cells were sometimes found central to the band in the lower layer under the region of the streak. In 9/18 stage XI-XII quail grafts and for the one stage X quail graft used, this contribution was assessed as +/–, indicating ten or less cells in the lower layer below the streak (Fig. 6D) (see Fig. 7A). In the remainder, the contribution was assessed as +, indicating that up to half the cells in the lower layer were derived from the graft (Fig. 6D) (see Fig. 7B for a result of this type). In six cases in which a streak was not induced, and in four cases in which a streak appeared from a lateral region (Fig. 7F), the distribution of quail cells was similar to that in cases that did form a streak (data not shown).

Six embryos with induced streaks were sectioned and the results confirmed the location and number of quail cells. See Fig. 7H for an example of an embryo with no cells under the streak.

(2) Grafts of posterior MZ including the sickle contribute cells to the primitive streak

As expected from our fate-mapping study, the largest contribution of quail cells occurred when the graft included the sickle. In most such cases, the whole lower layer under the area pellucida up to the germinal crescent was apparently formed by quail cells (++ in Fig. 6F), and there was often a small contribution of quail cells to the midline epiblast of the primitive streak (+++ in Figs 6F, 7C). Sections confirmed that the lower layer was formed by quail cells and demonstrated that some quail cells were scattered in the lower layers of the node and the middle layer of the streak (Fig. 7I).

(3) The distribution of quail cells is similar using grafts of anterior or posterior MZ

The distribution of quail cells in controls receiving a graft of anterior MZ did not differ significantly from that in experimental samples receiving a posterior MZ graft without the sickle (compare Fig. 6B and D). Again, a band of lower layer cells extended a short distance centripetally from the graft (Fig. 7G).

In conclusion, posterior MZ is able to induce a primitive streak without contribution to the streak, and with insignificant contribution to the lower layer (endoblast).

Formation of the ectopic organizer and primitive streak involves a change in fate of cells in the anterior region of the host

As shown above, when chick anterior halves are cultured alone, the streak that forms by regulation most often originates from the posterior region. To determine whether the ectopic streak adjacent to a quail graft of posterior MZ without the sickle arises from cells near the graft or from cells located near the

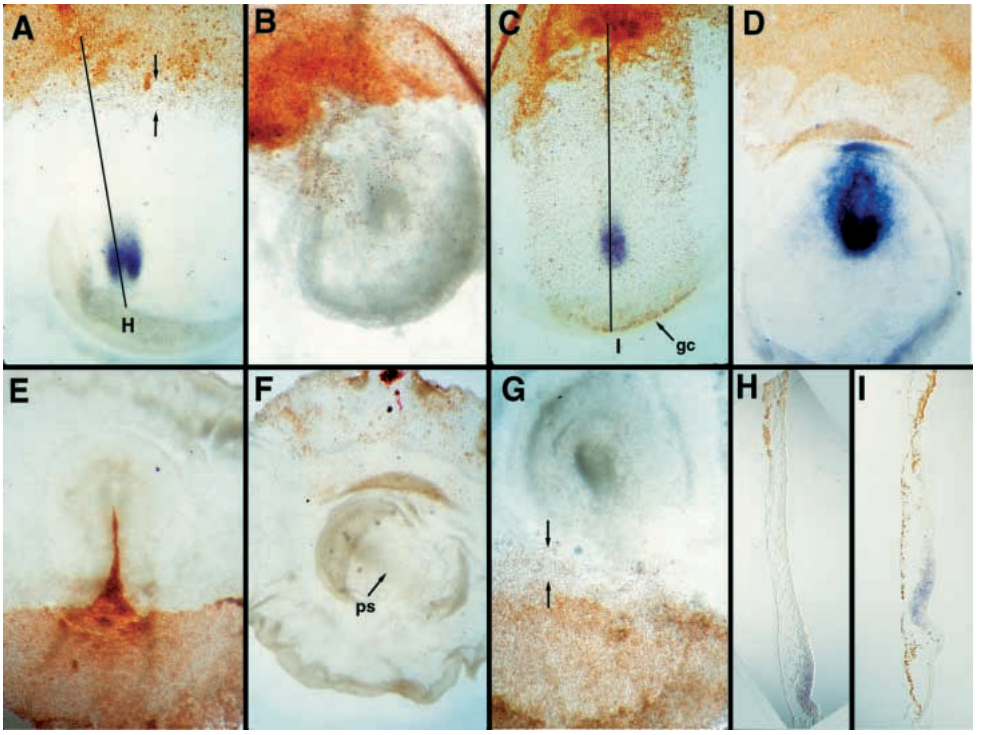


Fig. 7. Examples of the distribution of quail cells in whole mounts and sections of chick anterior halves fused with quail MZ after overnight incubation. The posterior side of the chick anterior half is down in all panels. Nuclei of quail cells appear brown after immunohistochemical staining with QCPN antibody. (A,C,H and I) Embryos hybridized to a *gsc* probe; (D) hybridized to a *Ch-T* probe. (A) Graft of posterior MZ without the sickle to the anterior side of a stage XI embryo. No quail cells are observed under the primitive streak region. Quail cells form a thin band (arrows) in the lower layer adjacent to the bulk of the quail piece in which both layers are quail cells. (B) Graft of posterior MZ cut through the sickle to the anterior side of a stage XII embryo. Less than half of the lower layer cells in the primitive streak region are quail cells. (C) Graft of posterior MZ with the sickle to the anterior side of a stage XI embryo. Most or all cells in the lower layer underlying the primitive streak region (endoblast) are quail cells. The hypoblast in the germinal crescent (gc) also contains quail cells. A few quail cells are present in the midline of the posterior end of the streak. (D) Graft of posterior MZ without the sickle to the anterior side of a stage XI embryo. About 10 quail cells were observed in the lower layer in the primitive streak region. (E) Dorsal view after grafting posterior MZ with the sickle to the posterior side of a stage XI embryo. Quail cells are present in the midline of the primitive streak almost up to the node. (F) Sample in which a posterior MZ cut through the sickle was grafted to the anterior side of a stage XI embryo. A host streak (arrow) originates from a lateral (right) region. The carmine mark on the quail graft is visible at the top. (G) A control sample in which a streak originates from the posterior side adjacent to the quail anterior MZ. Note the band of quail cells moving anteriorly in the lower layer (arrows). (H) Sagittal section after grafting posterior MZ without the sickle to the anterior side of a stage XI embryo. The plane of section is indicated for a similar embryo in A. At the site of the quail graft, all layers are composed of quail cells; adjacent to this is a region in which only the lower layer contains quail cells, and under the streak there are no quail cells. (I) Sagittal section of an embryo similar to that in C, where the plane of section is indicated. Quail cells make up the bulk of the lower layer, and a few are scattered in the node and streak.

posterior edge, the chick anterior region was labeled with carbocyanine dyes. The tissue near the posterior edge of the chick anterior half was labeled at three points with DiI (red), and one midline site near the quail graft was labeled with DiO (green) (Fig. 8A,B). In 4/4 cases in which streaks formed originating from the graft side, they included DiO-labeled cells, but did not contain significant DiI labeling (Fig. 8C,E). In contrast, when streaks formed from the lateral or posterior regions, DiI-labeled cells from the posterior edge were incorporated into them (not shown).

Thus, the ectopic streak does not form by recruiting cells from the posterior region of the anterior half; rather, the graft of posterior MZ is able to induce a change in fate of nearby cells from prospective extraembryonic and surface ectoderm (Hatada and Stern, 1994) to primitive streak.

Anterior halves of stage XII-XIII embryos are unable to respond to a graft of posterior MZ

At stage XII there was a sharp decline in the ability of host anterior halves to respond to a grafted posterior MZ by changing the position of the streak. Only 1/19 stage XII-XIII hosts formed a streak oriented from a graft without the sickle (Table 2B).

Grafts of posterior MZ to the posterior side of anterior halves increases the frequency of primitive streaks from the posterior pole

To confirm its streak-inducing properties, the posterior MZ was grafted to the posterior side of anterior halves (see Fig. 4 for the positioning of the graft). After grafting posterior MZ without the sickle, 95% formed a streak oriented from the graft (Table 2A, Fig. 6G), compared to 43% of streaks from the posterior pole in control grafts (Fig. 6A), a statistically significant increase, $P \leq 0.001$ (χ^2 test). After grafting posterior MZ with the sickle, as expected, the streak was oriented from the posterior pole in 95% of the cases (Table 2A).

In 8/14 cases in which posterior MZ without sickle was grafted, there was very little contribution of quail cells to the lower layer (+/– in Fig. 6H). After grafting posterior MZ with the sickle, a variable number of quail cells were found in the lower layer (data not shown), and 3/13 analyzed displayed quail cells in the midline epiblast of the primitive streak (see Fig. 7E), with a substantially larger contribution than was observed after transplants to the anterior side.

We conclude that a graft of posterior MZ without the sickle is able to increase the frequency of streak formation from the posterior side without contribution to the streak.

A *gsc*-expressing hypoblast is not necessary for streak formation

To clarify the role of the hypoblast in the formation of the streak, we explored whether a *gsc*-expressing hypoblast forms at early times under the area that will develop a newly positioned streak. 13 quail grafts without the sickle were placed next to the anterior side of chick stage XI-XII anterior halves and fixed before or during the initial stages of streak formation. For stage XI hosts fixed at 7 hours, 4/5 had a small amount of *gsc*-staining hypoblast under the original posterior side of the central disc (not shown), while 2/2 stage XII hosts had a distinct hypoblast on the original posterior side (Fig. 9A). Sections confirmed that quail cells remained near the graft and did not express *gsc* (Fig. 9E). By 13-17 hours, 6/6 stage XI and XII hosts had no *gsc*-staining hypoblast (Fig. 9B). Thus, no hypoblast was generated from the graft. Quail cells were found to have moved a short distance centripetally in the lower layer under the chick epiblast in both experimentals (Fig. 9A,E) and controls (Fig. 9C). Sections of experimental and control embryos showed no quail cells in the upper layer (e.g. Fig. 9E).

Eight quail grafts cut through or behind the sickle were placed next to the posterior side of chick stage XI anterior halves. In embryos fixed after 7 hours, some *gsc*-expressing hypoblast was apparent on the posterior side of the chick anterior half (Fig. 9D). At 13 hours, a variable amount of *gsc*-expressing hypoblast was present (not shown). Again, quail cells formed at most a narrow band in the lower layer adjacent to the graft.

In conclusion, after transplants to the anterior side, a *gsc*-expressing hypoblast is often present at early times on the posterior side (opposite to the site of probable streak formation). It presumably arises from islands and/or small pieces of hypoblast present in the anterior half, and disappears by 13-17 hours. A *gsc*-expressing hypoblast does not appear on the anterior side, demonstrating that it is not required during streak formation. In addition, we found no evidence for quail cells moving into the primitive streak, ruling out the possibility that the quail cells found at later stages in the lower layer are derived from the streak.

DISCUSSION

Induction of the organizer and primitive streak by the posterior MZ

Our main conclusion is that a graft of posterior MZ (without the sickle) is able to induce an organizer and primitive streak from the anterior pole without contribution to the streak. The ectopic streak is derived from host epiblast near the graft, rather than from cells migrating from a distance. The induced node and primitive streak are normal morphologically, expressing the streak marker *Ch-T* and the node markers *gsc* and *chordin* appropriately.

In contrast, grafts of posterior MZ with the sickle do contribute to the streak. By several criteria, these grafts are more potent than grafts without the sickle: they cause the formation of a streak when grafted to whole embryos, they cause the appearance of a streak at higher frequency when grafted to the anterior side of anterior halves, and grafts with the sickle are effective at stage XII-XIII, whereas competence to respond to the posterior MZ without the sickle is lost by stage XII. Our fate-mapping studies have confirmed that cells within the sickle, as well as epiblast dorsal to the sickle, contribute to the node and thus probably include organizer cells. Therefore, grafts including the sickle organize the ectopic node and streak, that is, cells from the grafts undergo self-differentiation and contribution to the axis, while also recruiting host cells to the axis.

Regulation in anterior halves and induction by the posterior MZ

Since control anterior halves are able to form a streak by regulation, our key result involves comparison of the orientation of streaks in control and experimental cases.

Anterior halves receiving a graft of posterior MZ without the sickle form a primitive streak oriented from the anterior pole in significantly more cases than in controls. The very low potential of the anterior zone to form a streak is perhaps best indicated by the fact that anterior quarters form a streak in only 4% of the cases compared to 81% for lateral quarters, and 96% for posterior quarters (Spratt and Haas, 1960b; see also Lutz, 1949).

In theory, a streak could arise from the anterior pole after grafting posterior MZ as a result of removal of an inhibitory influence of the anterior MZ rather than from a positive effect of the posterior MZ. However, several lines of evidence suggest that this is not the case. First, no streaks arise from the posterior side of isolated anterior halves (Spratt and Haas, 1960b), but the frequency of such streaks is 43% when an anterior MZ is placed next to the posterior side (our results). Second, 100% of posterior halves form streaks from their posterior side, indicating that an inhibitory influence from the anterior pole is not required to position the streak. Third, grafts of posterior MZ to the anterior side of whole embryos lacking anterior MZ do not result in appearance of an ectopic streak, indicating that the inhibitory effect is not coming from the anterior MZ. Thus, while inhibitory effects from the anterior MZ cannot be ruled out, this is not the primary means by which the position of the streak is determined.

We may view the chick embryo as composed of three regions: the posterior region where the streak normally forms, a middle zone where the streak forms if the posterior region is removed, and an anterior zone where the streak rarely forms in isolated halves or quarters. We have ruled out the possibility that the induced streak forms by recruitment of cells from the middle zone (the posterior region of the anterior half), where streaks usually form in isolated anterior halves. Our major finding is that the posterior MZ without the sickle greatly increases the probability that adjacent cells will form a streak in this anterior region, and thus acts as an inducer capable of changing the fate of adjacent cells from ectoderm to streak and node.

Factors expressed in the posterior MZ and induction of a primitive streak by secreted factors

The MZ posterior to the sickle expresses several factors at stages X-XII that could support inductive action. The epiblast of the posterior MZ expresses *cVg-1* (Seleiro et al., 1996) and *cWnt-8c* (Hume and Dodd, 1993). The Vg1/activin and Wnt pathways have been implicated in the function of the Nieuwkoop Center in Xenopus embryos (for a review, see Harland and Gerhart, 1997).

Recent evidence supports a role for these pathways in the chick embryo as well. Aggregates of mammalian cells secreting Wnt1 are able to promote streak formation in adjacent central disc cells (Cooke et al., 1994), and cells secreting cVg1 have a stronger effect (Seleiro et al., 1996; Shah et al., 1997). The cVg1-secreting cells stimulate expression of cVg1 in adjacent chick cells (I. Skromne, unpublished observations), and are effective only when placed on the MZ and not on the central disc (Shah et al., 1997), suggesting that the Vg1 effect is mediated by the MZ. cVg1-secreting pellets apparently have a stronger inducing activity than the posterior MZ itself, in that the pellet can induce a second axis in whole embryos at 180° to the host axis, and can induce an axis even if grafted up to stage XIII. These effects may be due to the high level of Vg1 emitted by the aggregates, and/or to stage-specific changes in the processing of endogenous cVg1.

It is plausible that Wnts, Vg1 and other factors expressed in the posterior MZ epiblast act upon neighboring host epiblast cells to promote initiation of streak formation, both in the experimental grafts and in normal development. In addition, a role for the deep layer of the posterior MZ in the inductive process cannot be excluded.

Fate mapping

We used DiI/DiO labeling to establish the fate of small groups of labeled cells in unmanipulated whole embryos to assess their normal fates in the most undisturbed conditions.

(1) The border of epiblast cells contributing to the streak and those remaining posterior to the streak lies just posterior to the sickle at stages XI-XIII

Labeled epiblast just posterior to the sickle remains in the posterior area opaca in two thirds of the samples from stages XI-XIII, while at stage X it contributes to the posterior streak. The epiblast above the middle of the sickle corresponds later in development to a position about halfway along the length of the primitive streak; this position in turn marks the boundary between future embryonic and extraembryonic mesoderm (Spratt and Haas, 1960b; Vakaet, 1970; Nicolet, 1971; Garcia-Martinez et al., 1993; Psychoyos and Stern, 1996). Thus, the epiblast posterior to the sickle never contains precursors of the region of the streak destined to give rise to embryonic structures. In addition, in agreement with Vakaet (1985), at prestreak stages the prospective extraembryonic mesoderm apparently lies in a narrow band just peripheral to the embryonic mesoderm. The contribution to the node of epiblast anterior to the sickle declined with time, consistent with the results of Hatada and Stern (1994), who found node precursors near the sickle at stages X-XI and more anteriorly by stage XIII.

(2) Distinct origins of hypoblast and endoblast in the lower layer

In agreement with results of earlier workers showing anterior displacement of the whole primary hypoblast (Spratt and Haas, 1960a; Vakaet, 1970; Stern, 1990), labeled cells in the hypoblast just anterior to the sickle move anteriorly to the germinal crescent by stage 4. Cells located slightly more posteriorly and ventral to the sickle appear in the endoblast under the central disc at stages 3-4. Consistent with these results, Callebaut and van Nueten (1994) and Callebaut et al. (1997) have shown that the endoblast underlying an ectopic streak derives from cells associated with the sickle.

Eyal-Giladi et al. (1992) labeled posterior pieces with or without the sickle with rhodamine-dextran-lysine and grafted them back to their site of origin in stage X-XI embryos, and found that the lower layer under the stage-4 primitive streak is derived from pieces including the sickle. They called the portion of the lower layer that is not primitive streak-derived endoderm hypoblast, while we call it endoblast. The results are consistent with ours, but their interpretation is that posterior MZ epiblast cells move ventrally into the sickle and then anteriorly into the hypoblast where they play an inductive role (Azar and Eyal-Giladi, 1979; Eyal-Giladi et al., 1992, 1994). However, the methods used do not permit an analysis of the

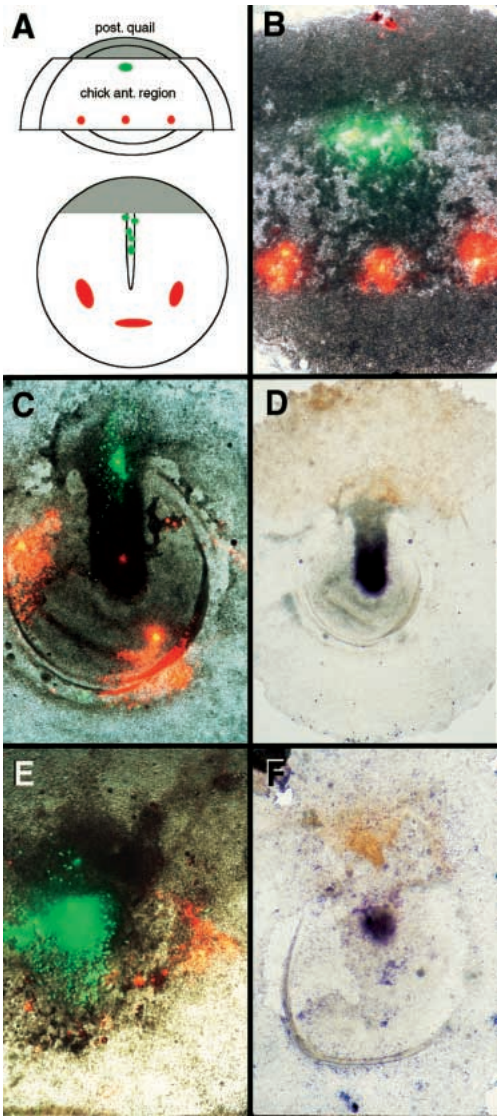


Fig. 8. A graft of quail posterior marginal zone to the anterior side of the chick anterior half changes the fate of nearby cells. (A) The upper diagram shows the sites of labeling of the chick anterior region at the start of incubation. Three clusters of cells along its posterior border are labeled with DiI (red), and one cluster in the midline near the anterior edge is labeled with DiO (green). A quail graft of posterior marginal zone is positioned on the anterior side. The lower diagram indicates the position of the labeled cells at the end of approximately 22 hours of incubation in cases in which a streak appeared from the posterior pole. (B) A grafted chick anterior half representing the labeling pattern with DiI and DiO at the initiation of culture. A red carmine marker is visible at the top in the middle of the quail piece. (C) An example of a case in which an ectopic streak appeared from the anterior pole during culture. DiI-labeled cells are found well outside the streak, and DiO-labeled cells are found in the streak. (D) The same embryo as in C, demonstrating expression of *chordin* in the node (purple) by in situ hybridization. Immunohistochemistry with QCPN antibody (brown) shows that quail cells did not contribute to the streak. (E) Another example in which an ectopic streak formed from the anterior pole. DiI-labeled cells are found well outside the central region and DiO-labeled cells form a dense cluster within the central region. (F) The same embryo as in E, demonstrating that the dense cluster of DiI-labeled cells corresponds to a *chordin*-expressing node. Quail cells remain posterior to the streak.

contribution of the different labeled layers to the resulting labeled structures. We find that in some cases posterior MZ epiblast contributed primarily to the posterior streak, but we cannot exclude that at the same time there was some contribution to the endoblast. In any case, these cells do not move anteriorly to the level of the anterior streak.

(3) The fate of grafted quail cells

The fate of quail posterior MZ with or without the sickle grafted to chick anterior halves was generally consistent with its normal fate. Grafts with the sickle were able to contribute to the streak, while grafts without did not. Labeling of hypoblast in the germinal crescent was rarely observed, confirming that the hypoblast is formed from cells located anterior to the sickle. Grafts including the sickle contributed somewhat less to the streak epiblast than expected from results in normal whole embryos, especially in the case of grafts to the anterior side. Grafts without the sickle also contributed less than expected to the primitive streak. Such grafts from donors at stages XI-XII never contributed to the posterior streak epiblast, while results from normal embryos would predict a contribution in about one quarter of the cases. Callebaut et al. (1996), using small homotopic grafts from prestreak quail embryos, have also observed that the posterior MZ does not contribute to the streak.

Grafts without the sickle contributed slightly more to the endoblast than expected from normal embryos, while chick lateral regions formed much more endoblast than expected, presumably compensating for the absence of the hypoblast and of the sickle, the normal source of endoblast.

Role of the lower layer

Our results indicate that a non-*gsc*-expressing endoblast forms under the streak and central disc as the streak appears and the hypoblast moves to the germinal crescent. The endoblast differs from the hypoblast in its smaller cells (Stern and Ireland, 1981), its growth pattern (Vakaet, 1970), its more posterior origin (our result) and its location at stage 4. It is distinguished from the hypoblast by the absence of expression of *gsc* (our results) or *otx2* (see Fig. 2 in Bally-Cuif et al., 1995).

We find that the appearance of a streak adjacent to a graft of posterior MZ without the sickle to the anterior side is not accompanied by outgrowth of hypoblast, defined as a *gsc*-expressing lower layer. Stern (1990) also found that a streak could form in the absence of hypoblast. Both examples demonstrate that the hypoblast is not required for formation of the primitive streak. However, the hypoblast has a positive effect since stage XIII central disc epiblast does not develop a streak, while central disc epiblast plus hypoblast (Azar and Eyal-Giladi, 1979) or disaggregated hypoblast (Mitrani and Eyal-Giladi, 1981) are able to form a streak. Thus, the hypoblast may play a permissive role, and may promote cell movements or other processes that contribute to streak formation.

In our grafted embryos, a non-*gsc*-expressing endoblast layer forms under the induced streak, composed mostly of chick host cells. It presumably forms by ingrowth of the deep layer of the lateral MZ, consistent with the finding of Stern and

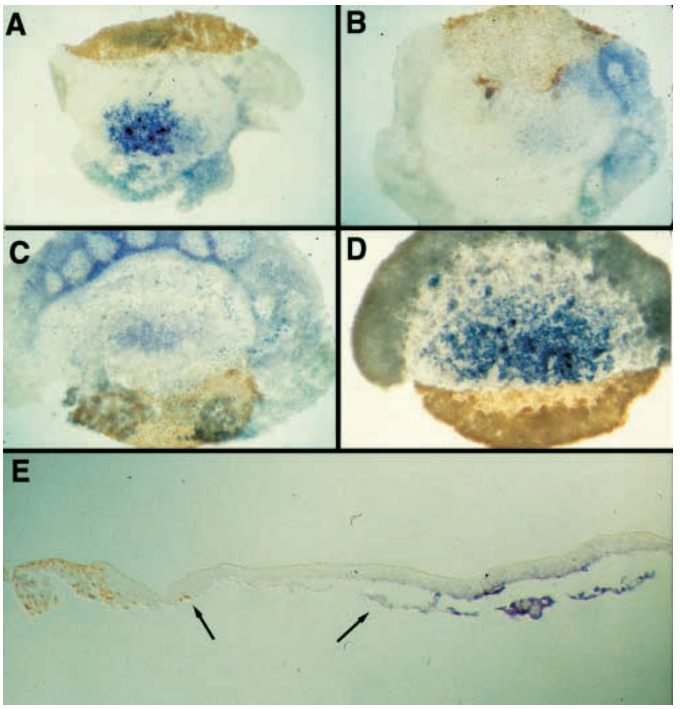


Fig. 9. Whole mounts of chick anterior halves fused with quail MZ and fixed at 7-15 hours. Samples were hybridized to a *gsc* probe and stained to reveal the quail QCPN antigen. The posterior side of the chick half is down in all panels. (A) A quail posterior MZ without the sickle was grafted to the anterior side of a stage XII chick anterior half and fixed at 7 hours. A *gsc*-expressing hypoblast is present at the original posterior side. Quail cells moving from the graft form a narrow band under the chick epiblast. (B) A quail posterior MZ cut through the sickle was grafted to the anterior side of a stage XI chick anterior half and fixed at 15 hours. No *gsc*-expressing hypoblast is present. (C) Control sample fixed at 13 hours. No *gsc*-expressing hypoblast is present. Quail cells moving from the graft form a narrow band under the chick epiblast. (D) A quail posterior MZ without the sickle was grafted to the posterior side of a stage XI chick anterior half and fixed at 7 hours of incubation. This sample had the most abundant *gsc*-positive hypoblast found in this set. (E) A section of the embryo in A. Arrows indicate the posterior edge of the quail cells migrating centrally in the lower layer (left), and the most anterior extent of the *gsc*-expressing hypoblast (right).

Ireland (1981) that the deep layers regardless of position tend to grow centripetally to replace a lower layer that has been removed. Since in our grafted embryos, there was a very small contribution of quail cells (10 or less cells) to the endoblast, it is clear that formation of an endoblast layer derived from the posterior region is not required for streak formation. The formation of a lower layer (hypoblast or endoblast) may be a critical permissive event, since grafted halves not forming a streak often had an incomplete lower layer.

Eyal-Giladi et al. (1994) placed strips of stage X posterior MZ placed on the ventral side of a stage XIII central disc epiblast and found that they were unable to induce a streak. The results were interpreted as indicating that the posterior MZ epiblast cells do not gain inductive power until they move ventrally and anteriorly into the hypoblast. However, we have shown directly that the posterior MZ (without the sickle) is able to induce a streak when placed adjacent to stage X-XI central disc cells, without formation of an inductive lower layer.

We conclude that neither a *gsc*-expressing hypoblast nor an endoblast layer derived from the posterior MZ is required for induction of the primitive streak and node.

Loss of competence at stage XII

At stage XI, anterior halves possess some polarity in that isolated halves are more likely to form a streak from the posterior than from other positions. By stage XII, the polarity is more distinct in that the anterior half is no longer able to respond to the posterior MZ graft and a streak from the posterior side becomes most likely in experimental as well as in control samples. The increasing polarization of the anterior half between stages XI and XII could be due to the movement of future organizer cells into the anterior half by stage XII (Hatada and Stern, 1994). It may also be promoted by the spread of inductive influences from the posterior MZ and/or hypoblast, reflected in graded gene expression reaching into the anterior half of the epiblast, such as *cNot1*, *cNot2* and *otx2* (Knezevic et al., 1995; Stein et al., 1996; Bally-Cuif et al., 1995). A third possibility is that older embryos are under increasingly strong inhibitory influences, but our results suggest that these are not long-range signals from the posterior (streak-forming) region.

Conclusion

The posterior MZ of the chick embryo has strong similarities to the Nieuwkoop Center of the amphibian embryo, the dorsal vegetal center described by Nieuwkoop as capable of inducing an organizer in adjacent animal cells without contributing to it. The posterior MZ occupies a position that may be homologous to that of the Nieuwkoop Center (Bachvarova, 1996) and it expresses genes homologous to those implicated in Nieuwkoop Center function, such as *Vg1* and *Wnt8c*. Most important, it is able to induce a node and streak in adjacent cells without contributing cells to these structures. It remains to be determined whether induction by the posterior MZ is required during normal streak formation or whether such induction acts in parallel with autonomous processes within the epiblast that are sufficient for streak formation.

We wish to thank John Gerhart, Jonathan Slack and Andrea Streit for critical reading of the manuscript, and Jim Smith for providing the *Ch-T* plasmid. This study was supported by the Human Frontiers Science Program, the National Institutes of Health (C. D. S.) and DGAPA, UNAM, Mexico (I. S.). The work was carried out while R. F. B. was on sabbatical leave from Cornell University Medical College in the laboratory of C. D. Stern.

REFERENCES

Azar, Y. and Eyal-Giladi, H. (1979). Marginal zone cells - the primitive streak-inducing component of the primary hypoblast in the chick. *J. Embryol. exp. Morph.* **52**, 79-88.

Azar, Y. and Eyal-Giladi, H. (1981). Interaction of epiblast and hypoblast in the formation of the primitive streak and the embryonic axis in chick, as revealed by hypoblast-rotation experiments. *J. Embryol. exp. Morph.* **61**, 133-144.

Bachvarova, R. F. (1995). Anterior-posterior polarization and mesoderm inducing factors in the pregastrula mouse embryo: Comparison to chick and frog embryos. In *Adv. Dev. Biol.* vol. 4 (ed. P. Wassarman), pp. 147-189. Jai Press, Greenwich, CT.

Bally-Cuif, L., Gulizano, M., Broccoli, V. and Boncinelli, E. (1995). *c-otx2*

is expressed in two different phases of gastrulation and is sensitive to retinoic acid treatment in chick embryo. *Mech. Dev.* **49**, 49-63.

Bellairs, R., Bromham, D. R. and Wylie, C. C. (1967). The influence of the area opaca on the development of the young chick embryo. *J. Embryol. exp. Morph.* **17**, 195-212.

Boterenbrood, E. C. and Nieuwkoop, P. D. (1973). The formation of the mesoderm in Urodelean amphibians. V. Its regional induction by the endoderm. *Wilhelm Roux's Arch. EntwichMech. Org.* **173**, 319-332.

Callebaut, M. and van Nueten, E. (1994). Rauber's (Koller's) sickle: The early gastrulation organizer of the avian blastoderm. *Eur. J. Morph*. **32**, 35-48.

Callebaut, M., van Nueten, E., Bortier, H., Harrisson, F. and van Nassauw, L. (1996). Map of the anlage fields in the avian unincubated blastoderm. *Eur. J. Morph.* **34**, 347-361.

Callebaut, M., van Nueten, E., Harrisson, F., van Nassauw, L., Schrevens, A. and Bortier, H. (1997). Avian gastrulation and neurulation are not impaired by the removal of the marginal zone at the unincubated blastoderm stage. *Eur. J. Morph.* **35**, 69-77.

Cooke, J., Takada, S. and McMahon, A. (1994). Experimental control of axial pattern in the chick blastoderm by local expression of Wnt and activin: The role of HNK-1 positive cells. *Dev. Biol.* **164**, 513-527.

Eyal-Giladi, H. and Kochav, S. (1976). From cleavage to primitive streak formation: A complementary normal table and a new look at the first stages of the development of the chick. I. General morphology. *Dev. Biol*. **49**, 321-337.

Eyal-Giladi, H. and Khaner, O. (1989). The chick's marginal zone and primitive streak formation. II. Quantification of the marginal zone's potencies - temporal and spatial aspects. *Dev. Biol.* **134**, 215-221.

Eyal-Giladi, H., Debby, A. and Harel, N. (1992). The posterior section of the chick's area pellucida and its involvement in hypoblast and primitive streak formation. *Development* **116**, 819-830.

Eyal-Giladi, H., Lotan, T., Levin, T., Avner, O. and Hochman, J. (1994). Avian marginal zone cells function as primitive streak inducers only after their migration into the hypoblast. *Development* **120**, 2501-2509.

Garcia-Martinez, V., Alvarez, I. S. and Schoenwolf, G. C. (1993). Locations of the ectodermal and nonectodermal subdivisions of the epiblast at stages 3 and 4 of avian gastrulation and neurulation. *J. Exp. Zool.* **267**, 431-446.

Gerhart, J., Black, S., Scharf, S., Gimlich, R., Vincent, J.-P., Danilchik, M., Rowning, B. and Roberts, J. (1986). Amphibian early development. *Biosci.* **36**, 541-549.

Gimlich, R. L. and Gerhart, J. C. (1984). Early cellular interactions promote embryonic axis formation in *Xenopus laevis*. *Dev. Biol.* **104**, 117-130.

Gimlich, R. L. (1986). Acquisition of developmental autonomy in the equatorial region of the Xenopus embryo. *Dev. Biol.* **115**, 340-352.

Hamburger, V. and Hamilton, H. L. (1951). A series of normal stages in the development of the chick. *J. Morph.* **88**, 49-92.

Harland, R. and Gerhart, J. (1997). Formation and function of Spemann's organizer. *Annu. Rev. Cell Dev. Biol.* **13**, 611-667.

Hatada, Y. and Stern, C. D. (1994). A fate map of the epiblast of the early chick embryo. *Development* **120**, 2879-2889.

Hume, C. R. and Dodd, J. (1993). *Cwnt-8C*: A novel Wnt gene with a potential role in primitive streak formation and hindbrain organization. *Development* **119**, 1147-1160.

Izpisua-Belmonte, J. C., De Robertis, E. M., Storey, K. G. and Stern, C. D. (1993). The homeobox gene *goosecoid* and the origin of organizer cells in the early chick blastoderm. *Cell* **74**, 645-659.

Khaner, O. (1995). The rotated hypoblast of the chicken embryo does not initiate an ectopic axis in the epiblast. *Proc. Nat. Acad. Sci. USA* **92**, 10733-10737.

Khaner, O. (1998). The ability to initiate an axis in the avian blastula is concentrated mainly at a posterior site. *Dev. Biol*. **194**, 257-266.

Khaner, O. and Eyal-Giladi, H. (1986). The embryo-forming potency of the posterior marginal zone in stages X through XII of the chick. *Dev. Biol.* **115**, 275-281.

Khaner, O. and Eyal-Giladi, H. (1989). The chick's marginal zone and primitive streak formation. I. Coordinate effect of induction and inhibition. *Dev. Biol.* **134**, 206-214.

Knezevic, V., Ransom, M. and Mackem, S. (1995). The organizer-associated chick homeobox gene, *Gnot1*, is expressed before gastrulation and regulated synergistically by activin and retinoic acid. *Dev. Biol.* **171**, 458-470.

Lutz, H. (1949). Sur la production expérimentale de la polyembryonie et de la monstruosité double chez les oiseaux. *Arch. Anat. Microsc. Morph. Exp.* **38**, 79-144.

Mitrani, E. and Eyal-Giladi, H. (1981). Hypoblastic cells can form a disk inducing an embryonic axis in chick epiblast. *Nature* **289**, 800-802.

Mitrani, E., Ziv, T., Thomsen, G., Shimoni, Y., Melton, D. A. and Bril, A. (1990). Activin can induce the formation of axial structures and is expressed in the hypoblast of the chick. *Cell* **63**, 495-501.

New, D. A. T. (1955). A new technique for the cultivation of the chick embryo in vitro. *J. Embryol. exp. Morph.* **3**, 326-331.

Nicolet, G. (1971). Avian gastrulation. *Adv. Morph.* **9**, 231-262.

Nieuwkoop, P. D. (1969). The formation of the mesoderm in urodelean amphibians. II. The origin of the dorso-ventral polarity of the mesoderm. *Wilhelm Roux's Arch. EntwichMech. Org.* **163**, 298-315.

Nieuwkoop, P. D. (1977). Origin and establishment of embryonic polar axes in amphibian development. *Curr. Topics Dev. Biol.* **11**, 115-132.

Pannett, C. A. and Compton, A. (1924). The cultivation of tissues in saline embryonic juice. *Lancet* **206**, 381-384.

Peter, K. (1938). Untersuchungen uber die Entwicklung des Dotterendoderms. I. Die Entwicklung des Entoderms bein Huhnchen. *Z. Mikr. anat. Forschung* **43**, 362-415.

Psychoyos, D. and Stern, C. D. (1996). Fates and migratory routes of primitive streak cells in the chick embryo. *Development* **122**, 1523-1534.

Ruiz-i-Altaba, A., Placzek, M., Baldassare, M., Dodd, J. and Jessell, T.M. (1995). Early stages of notochord and floor plate development in the chick embryo defined by normal and induced expression of HNF-3β. *Dev. Biol.* **170**, 299-313.

Seleiro, E. A. P., Connolly, D. J. and Cooke, J. (1996). Early developmental expression and experimental axis determination by the chicken *Vg1* gene. *Curr. Biol.* **6**, 1476-1486.

Shah, S. B., Skromne, I., Hume, C. R., Kessler, D. S., Lee, K. J., Stern, C. D. and Dodd, J. (1997). Misexpression of chick Vg1 in the marginal zone induces primitive streak formation. *Development* **124,** 5127-5138.

Slack, J. M. W. (1994). Inducing factors in *Xenopus* early embryos. *Curr. Biol.* **4**, 116-126.

Spemann, H. and Mangold, H. (1924). Induction of embryonic primordia by implantation of organizers from a different species. In *Foundations of Experimental Embryology* (1974) (ed. B. H. Willier and J. M. Oppenheimer), pp. 144-184. Hafner Press, New York.

Spratt, N. T. and Haas, H. (1960a). Morphogenetic movements in the lower surface of the unincubated and early chick blastoderm. *J. Exp. Zool.* **144**, 139-158.

Spratt, N. T. and Haas, H. (1960b). Integrative mechanisms in development of the early chick blastoderm. I. Regulative potentiality of separated parts. *J. Exp. Zool.* **145**, 97-137.

Stein, S., Niss, K. and Kessel, M. (1996). Differential activation of the clustered homeobox genes *CNOT2* and *CNOT1* during notogenesis in the chick. *Dev. Biol.* **180**, 519-533.

Stern, C. D. (1990). The marginal zone and its contribution to the hypoblast and primitive streak of the chick embryo. *Development* **109**, 667-682.

Stern, C. D. and Ireland, G. W. (1981). An integrated experimental study of endoderm formation in avian embryos. *Anat. Embryol.* **163**, 245-263.

Streit, A., Sockanathan, S., Perez, L., Rex, M., Scotting, P. J., Sharpe, P. T., Lovell-Badge, R. and Stern, C. D. (1997). Preventing the loss of competence for neural induction: HGF/SF, L5 and *Sox-2*. *Development* **124**, 1191-1202.

Streit, A., Lee, K. J., Woo, I., Roberts, C., Jessell, T. M. and Stern, C. D. (1998). Chordin regulates primitive streak development and the stability of induced neural cells, but is not sufficient for neural induction in the chick embryo. *Development* **125**, 507-519.

Vakaet, L. (1970). Cinephotomicrographic investigations of gastrulation in the chick blastoderm. *Arch. Biol.* **81**, 387-426.

Vakaet, L. (1985). Morphogenetic movements and fate maps in the avian blastoderm. In *Molecular Determinants of Animal Form* (ed. G. M. Edelman), pp. 100-109. A.R. Liss, New York.

Waddington, C. H. (1932). Experiments on the development of chick and duck embryos, cultivated in vitro. *Phil. Trans. Roy. Soc. B.* **211**, 179-230.

Waddington, C. H. (1933). Induction by endoderm in birds. *Wilhelm. Roux's Arch. EntwichMech. Org.* **128**, 502-521.