

The marginal zone and its contribution to the hypoblast and primitive streak of the chick embryo

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Summary

The marginal zone of the chick embryo has been shown to play an important role in the formation of the hypoblast and of the primitive streak. In this study, time-lapse filming, fate mapping, ablation and transplantation experiments were combined to study its contribution to these structures. It was found that the deep (endodermal) portion of the posterior marginal zone contributes to the hypoblast and to the junctional endoblast, while the epiblast portion of the same region contributes to the epiblast of the primitive streak and to the definitive (gut) endoderm derived from it. Within the deep part of the posterior marginal zone, a subpopulation of HNK-1-positive cells contributes to the hypoblast.

Removal of the deep part of the marginal zone prevents regeneration of the hypoblast but not the

formation of a primitive streak. Removal of both layers of the marginal zone leads to a primitive streak of abnormal morphology but mesendodermal cells nevertheless differentiate.

These results show that the two main properties of the posterior marginal zone (contributing to the hypoblast and controlling the site of primitive streak formation) are separable, and reside in different germ layers. This conclusion does not support the idea that the influence of the posterior marginal zone on the development of axial structures is due to it being the source of secondary hypoblast cells.

Key words: hypoblast, germ layers, gastrulation, endoderm, cell movement, primitive streak, epiblast.

Introduction

The marginal zone of the pre-primitive-streak chick blastodisc is a ring that separates the inner, embryonic *area pellucida* from the outer, extraembryonic *area opaca* (see below under 'Explanation of terminology' and Figs 1, 2). Its importance during gastrulation was demonstrated long ago (Graeper, 1929; Mehrbach, 1935; Rudnick, 1935; Spratt and Haas, 1960*b,c*; Vakaet, 1970; see also Bellairs, 1986). Spratt and Haas (1960*b*) showed that fragments of blastoderm that contained marginal zone had a greater capacity to produce complete embryos than fragments that did not contain this region. Moreover, Spratt and Haas (1960*b*) demonstrated that the embryo-forming potential of the marginal zone increased in a graded way towards the posterior (caudal) end of the ring. The importance of the marginal zone, and in particular the special nature of the posterior margin, was confirmed in a more detailed series of experiments by Eyal-Giladi and her colleagues (Eyal-Giladi and Spratt, 1965; Azar and Eyal-Giladi, 1979; 1981; Mitrani *et al.* 1983; Khaner *et al.* 1985; Khaner and Eyal-Giladi, 1986; 1989; Eyal-Giladi and Khaner, 1989). They found, for example, that anteroposterior rotation of the marginal zone can reverse the craniocaudal orientation of the embryonic

axis, in the same way as Waddington (1933) had shown for rotation of the hypoblast. They also found that in the absence of the posterior marginal zone, an embryo is not capable of regenerating its hypoblast. Eyal-Giladi and co-workers have therefore argued that the posterior marginal zone contributes cells to the hypoblast and that it is this marginal zone derived hypoblast that induces the formation of the primitive streak and which prevents secondary axes from forming in the normal embryo.

Despite the fact that the importance of the marginal zone has been recognized for some time, the fate of its cells has received less experimental attention than those of other tissues at the same stage of development. It consists of a one-cell thick epithelium continuous with the epiblast of the *area pellucida* and of the *area opaca*, covered ventrally by a flap of tissue that is continuous with the yolky cells of the *area opaca* endoblast (germ wall; see **Explanation of terminology** below). Furthermore, some of the deep cells of the posterior (but not elsewhere) marginal zone stain with monoclonal antibody HNK-1 (Canning and Stern, 1988), suggesting that the deep (endoblast) portion of the marginal zone is itself complex, and that it contains more than one cell type.

It is known that the hypoblast is derived from two

distinct cellular contributions: first, at about the time of laying (stages IX–X; Eyal-Giladi and Kochav, 1976), separate islands of cells are found scattered over the ventral surface of the epiblast; they appear to arise by ingression of cells from the latter germ layer. The hypoblast forms a layer later in development (stages XI–XIII) as the islands receive a contribution from cells derived from the posterior margin. It is not yet known whether these cells arise within the marginal zone by ingression from the epiblast (but see Fraser, 1954).

In many previous transplantation and ablation experiments involving the marginal zone, the entire thickness of this region is extirpated and/or transplanted. It has therefore been impossible to determine which of the layers that constitute this region is responsible for its two major demonstrated roles during development: a source of hypoblast cells and a role in determining the position and orientation of the primitive streak. It has always been assumed, albeit without direct experimental evidence, that these two properties are linked, the hypoblast derived from this region being responsible for induction of the primitive streak.

In this study, detailed fate mapping of the marginal zone was undertaken by various techniques to determine the contribution of each of the germ layers to the hypoblast and primitive streak. It was found that the HNK-1-positive cells of the deep component of the posterior marginal zone are progenitors of the hypoblast; these cells migrate faster than the cells of the islands of hypoblast that arise earlier in development. By contrast, the cells of the epiblast component of the posterior marginal zone contribute to the epiblast portion of the primitive streak and to the definitive (gut) endoderm that is derived from the streak. I suggest that the contribution of the posterior marginal

zone to the hypoblast and its ability to control the site of formation and polarity of the primitive streak are separate properties, contained in different germ layer components of the marginal zone.

Explanation of terminology used

There is some confusion in the literature about various terms used to describe different regions and tissues of the early chick embryo. This section defines the terms used in this paper.

Components of the lower layer (Fig. 1)

In the nomenclature used here (see Bellairs, 1986), tissues that only contribute to extraembryonic structures (such as the yolk sac stalk) are denoted by the suffix *-blast* (e.g. hypoblast, junctional endoblast), while those that contribute to embryonic structures (such as the gut) have the suffix *-derm* (e.g. definitive endoderm). The islands of cells present at Eyal-Giladi and Kochav (1976) stage X–XI and which are probably derived by ingression from the epiblast are referred to as the *primary hypoblast* because it is the earliest component of the hypoblast to appear. The hypoblast sheet is completed by the addition of a second component, called the *secondary hypoblast*, which is derived from the posterior part of the embryo. The secondary hypoblast consists of the first cells to emerge from the posterior margin. As will be discussed in this paper, the cells of the primary and secondary hypoblast may mix with each other to some extent. Both components of the hypoblast eventually give rise only to extraembryonic endoblast in the yolk sac stalk.

After the appearance of the primitive streak, more

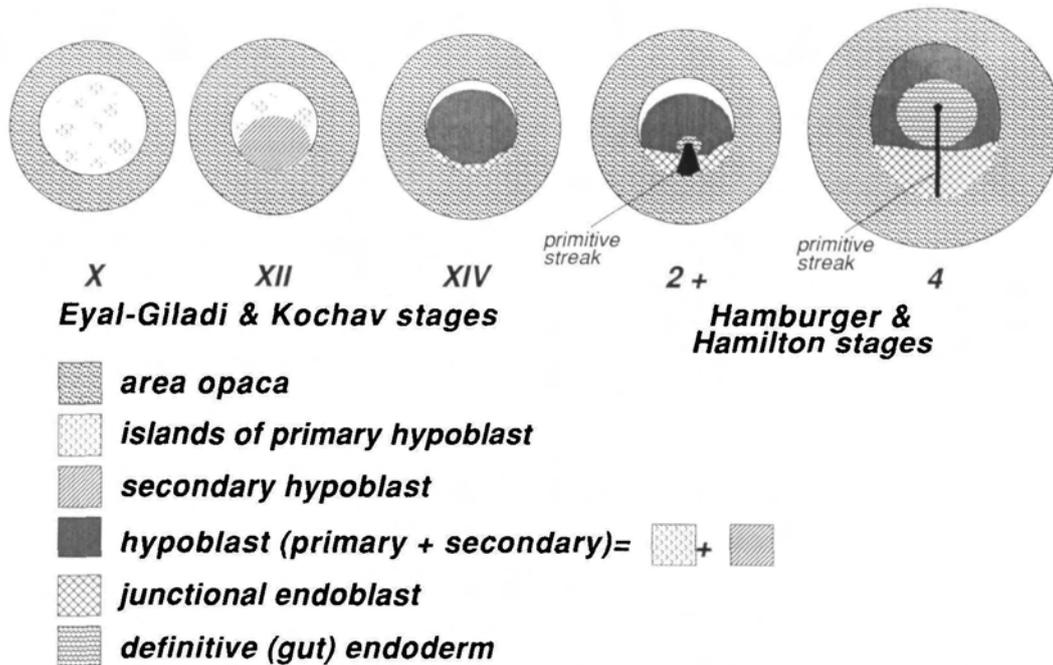


Fig. 1. Diagram explaining the terminology used to describe the components of the lower (endodermal) layer of chick embryos at stages X–4. In all the diagrams, the anterior (cephalic) end of the embryo is shown towards the top of the figure.

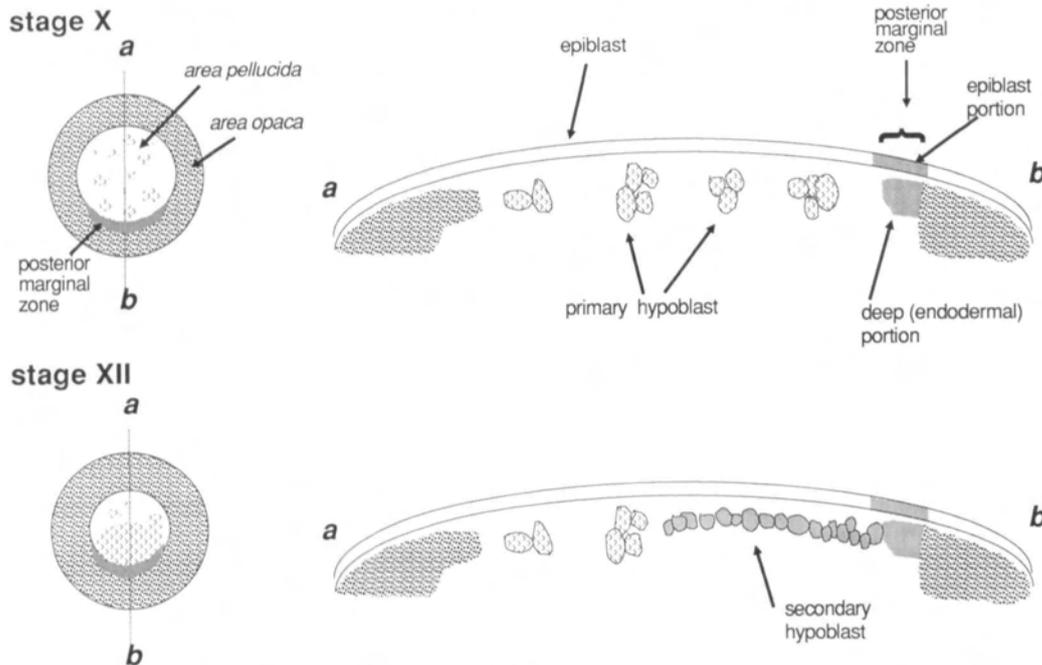


Fig. 2. Diagram explaining the terminology used to describe the components of the marginal zone of chick embryos at stages X and XII. The diagrams on the left represent whole embryos viewed from the ventral side, and those on the right represent sections passing through the plane a–b, where a is the anterior (cephalic) end of the embryo and b is the posterior (caudal) end. The posterior marginal zone is shown as a crescent for clarity, but the marginal zone is a ring that extends around the entire circumference of the blastoderm.

tissues are added: further cells emerge from the posterior margin to form the *junctional endoblast* (Vakaet, 1970), and some cells derived from the primitive streak itself insert into the centre of the hypoblast sheet, especially near the anterior (cranial) tip of the streak. This new central component of smaller and less yolky cells than those of the hypoblast (Bellairs *et al.* 1981; Stern and Ireland, 1981) is the *definitive (or gut) endoderm* (Bellairs, 1953). Eventually, the definitive endoderm displaces the hypoblast cells to the periphery of the *area pellucida*; at the anterior end of the embryo the hypoblast forms the *germinal crescent*, which may contain the primordial germ cells (see Bellairs, 1986).

The marginal zone (Fig. 2)

When Eyal-Giladi and her colleagues refer to the marginal zone (MZ), they have removed a fairly loose flap of *area opaca* endoblast (*germ wall*) tissue which underlies it on the ventral side. However, this flap of tissue is different from the rest of the *area opaca* endoblast; it is not attached to the epiblast as firmly as are more peripheral regions of the germ wall, and its posterior portion contains a population of HNK-1-positive cells (Canning and Stern, 1988). In embryos from which the flap has not been removed, it is found that its cells are continuous with those of the hypoblast (see Fig. 3 of Canning and Stern, 1988).

The *area opaca* endoblast (*germ wall*) therefore consists of at least two regions: a central ring, which I will call the *deep portion of the marginal zone*, and a peripheral ring, the *germ wall (or area opaca endoblast)*

proper. Both contain large yolky cells but can be distinguished by the presence of HNK-1-positive cells in the posterior margin and by the lack of attachment of the marginal region to the overlying epiblast. As will be shown in this paper, they can also be distinguished because the deep portion of the marginal zone contributes cells to the (secondary) hypoblast and junctional endoblast, while the cells of the germ wall proper move centrifugally. The deep portion of the marginal zone and the germ wall are continuous with the yolk underlying the embryo. However, explantation of the embryo usually separates the yolk from the *area opaca*.

The region of epiblast overlying the deep portion of the marginal zone is the region that I will refer to here as the *superficial portion of the marginal zone*. This is the region that Eyal-Giladi and her colleagues consider as the whole marginal zone. The superficial portion of the marginal zone can be distinguished from the rest of the epiblast by its lucent appearance and because in histological sections the cells are more cuboidal than those of the central epiblast, which are cylindrical or prismatic. At its posterior extremity, the superficial portion of the marginal zone displays a crescent shaped region, Köller's sickle (Köller, 1882); it is this region that has been shown to be important for the formation of the primitive streak.

Materials and methods

Embryo techniques

Young chick embryos at stages X–XII (pre-primitive streak

stages in Roman numerals according to Eyal-Giladi and Kochav, 1976; later stages in Arabic numerals according to Hamburger and Hamilton, 1951) were obtained from fertilized hens' eggs that had been incubated up to 6 h at 38°C. The embryos were explanted in Pannett-Compton saline (Pannett and Compton, 1924) and cultured according to the technique described by New (1955) with minor modifications (Stern and Ireland, 1981). The dishes containing the explanted embryos were incubated in a humidified atmosphere at 37°C until required (usually 12–24 h, by which time they had reached stages 3–5).

Time-lapse video microscopy

For time-lapse video filming, the embryo culture technique was modified further. Culture chambers were constructed from Cooper dishes (Falcon) by cutting a circular hole (about 25 mm diameter) in the centre of the lid and a similar hole in the centre of the base of the dish. Each hole was then covered with a glass coverslip (N°1.5, 32 mm diameter, Chance Proper), which was attached to the plastic dish with a thin layer of High Vacuum Silicone grease (BDH). The vitelline membrane carrying the embryo was stretched around a very thin (1 mm) ring (about 28 mm diameter) cut from glass tubing, and placed over a shallow pool of thin egg albumen. The inside surface of the lid, including the attached coverslip, were then coated with a thin layer of egg albumen and sealed onto the base. This assembly, diagrammatically shown in Fig. 3, allowed even high power microscope objectives to approach the embryo sufficiently to focus onto its ventral surface, which faced uppermost.

The culture chamber containing the embryo was placed onto the stage of an Olympus Vanox-T microscope surrounded with a chamber which was kept at $37.5 \pm 0.5^\circ\text{C}$ by means of a thermostatically controlled air curtain incubator. Hysteresis was built into the control circuit to lengthen the period of temperature oscillations in the chamber to about 45 s. Because the culture dishes were sealed with egg albumen, it was not necessary to humidify the warm chamber, which avoided condensation problems on the microscope objective.

The embryo was viewed with either bright-field, Nomarski differential interference contrast or angled reflected illumination from a fibre optics light source. Images were collected using a Silicon Intensifier Target (SIT) video camera (Custom Camera Designs, Wells, model LT1160-SIT) with automatic

or manual gain control and the possibility of separate control of tube voltage and intensifier voltage, and an electronic zoom (8:1)/pan facility. The signal from the SIT camera was fed into the analog input port of a Seescan I3000 (digitiser, frame store and image analyser) at a resolution of 256×256 pixels and 128 grey levels. A brief program was written to average 8 digitized frames. This procedure was carried out repeatedly, at a preset time interval depending on the objective used (lower power objectives, longer interval). After frame averaging, the image analysing computer was made to close a relay which activated a 'single shot' toggle of a Mitsubishi HS-480E time-lapse VHS video recorder. Closing this contact makes the video recorder store 4 identical frames and then pause until the next pulse from the Seescan computer. In some film sequences, contrast enhancement (grey level stretching) and/or γ -correction (non-linear contrast enhancement) was performed on each averaged frame prior to storage on video tape.

The video films made in this way were played back at video rate and normally viewed directly on a Philips video monitor. In some cases, however, the images were viewed through the Seescan computer, which allowed further contrast enhancement and frame-by-frame analysis of the images. Output from the Seescan was redirected to a RGB video monitor via a Mitsubishi CP100B Colour Video Copy Processor, which allowed rapid monochrome or pseudocolour prints to be made directly.

Fate mapping with carbocyanine dyes, DiI and DiO

Two different methods of labelling were used for applying the carbocyanine dyes 1,1'-dioctadecyl-3,3,3',3'-tetramethyl indocarbocyanine perchlorate (Molecular Probes, Inc.) (DiI) and 3,3'-dioctadecyloxycarbocyanine perchlorate (Molecular Probes Inc. Di-O-C18-(3)) (DiO). These intensely fluorescent dyes are completely insoluble in water but soluble in lipid and organic solvents, they are incorporated into cell membranes and are not transferred between cells (Honig and Hume, 1989; Serbedzija *et al.* 1989; Wetts and Fraser, 1989). DiI has peak excitation at 547 nm and emits at 571 nm, while DiO is excited at 484 nm and emits at 507 nm. Since both dyes are excited by 488 nm light, a broad band pass filter (allowing wavelengths longer than 490 nm to pass) allows both dyes to be discriminated with a single excitation wavelength under fluorescein optics.

In one series of 18 embryos, a crystal of each dye was placed directly onto the regions to be labelled of an embryo explanted in New (1955) culture using fine needles made from A1 insect pins. The crystals were applied when most of the fluid from the ventral surface of the embryo had been removed and there was only a thin layer of saline over the embryo.

In a second series of 54 embryos, a fine micropipette was pulled from a 50 μl glass capillary tube and used to deliver minute droplets of a solution (0.25% w/v of the dye, made up either in dimethylformamide or in 5% dimethylsulphoxide [DMSO] in absolute ethanol) of each of the dyes by applying gentle air pressure. This system was used to label small groups of cells (about 10–50 cells) in the embryo. Two control series of 3 embryos each were given either dimethylformamide or 5% DMSO in ethanol, respectively, injected into many locations in the embryo by the same technique. All of these embryos developed normally and no deleterious effects of the solvents were seen. One of the embryos injected at stage XI with DiI and DiO solutions in DMSO/ethanol in the primary hypoblast and deep posterior marginal zone, respectively, was filmed by time-lapse video microscopy. The labelled cells

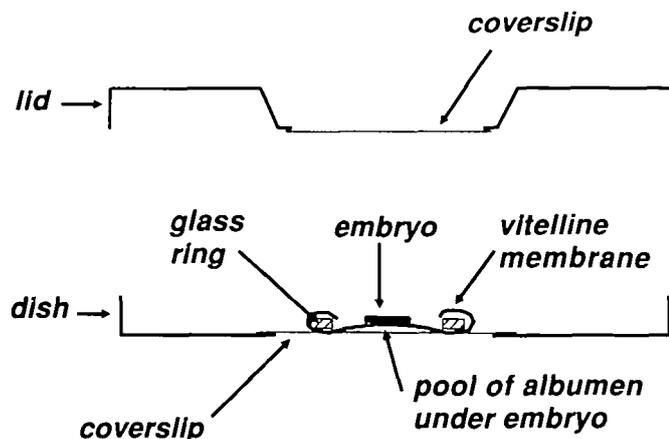


Fig. 3. Diagram of the assembly used to culture embryos for time-lapse filming.

could be identified without fluorescence; they appeared to behave identically to their unlabelled neighbours.

Lack of transfer of the dye between labelled cells was confirmed by observation of those embryos that had received DiI and DiO in adjacent regions of the same germ layer. No double-labelled cells were seen. Further confirmation of this was obtained by labelling two small groups of cells, one with each dye, in the hypoblast of an embryo that had been fixed in buffered formol saline for 30 min; neither dye had spread after incubation of the fixed embryo for 18 h at 37°C.

After labelling by either method, the experimental embryos were incubated for 12–24 h at 37°C and then fixed in 4% buffered formol saline (pH 7.0) containing 0.25% glutaraldehyde; they were mounted in the fixative. The embryos were then examined as soon as possible as a whole mount by epifluorescence microscopy in a Vanox-T microscope or by confocal microscopy using a BioRad Lasersharp MRC-500.

In some additional embryos labelled with DiI as described above, the germinal layer localization of labelled cells was confirmed by histology. However, it is not possible to section DiI-labelled embryos directly because drying and organic solvents cause the dye to spread away from the labelled cells. The labelled embryos were therefore processed prior to sectioning with a technique (Maranto, 1982; Buhl and Lübke, 1989) that uses the fluorescence emitted by the DiI to oxidize 3,3'-diaminobenzidine (DAB), producing an insoluble black product in the DiI labelled cells. Labelled embryos were fixed in 4% buffered formol saline containing 0.25% glutaraldehyde as described above, washed twice in 0.1 M Tris-HCl (pH 7.4), placed in a cavity slide immersed in a solution of 500 µg ml⁻¹ DAB in the same buffer and covered with a coverslip. The assembly was placed on the microscope under a 10× or 20× microscope objective and illuminated for epifluorescence appropriate for DiI excitation (see above), and exposed to light for about 1.5 h or until all the fluorescence had disappeared; the microscope was focussed every 10–15 min during the incubation. After incubation, the embryos were washed in tap water, dehydrated through alcohols, counterstained lightly in Fast Green and cleared in cedarwood oil before being embedded in Paraplast and sectioned conventionally (10–12 µm).

Fate mapping with HNK-1^{Au}

In order to investigate the fate of the HNK-1-positive cells of the posterior marginal zone, HNK-1 antibody was coupled to 15 nm colloidal gold (HNK-1^{Au}) and used as described by Stern and Canning (1990). The method for coupling was modified from Goodman *et al.* (1981); the optimal pH for coupling was found to be 8.2. Briefly, donor embryos at stage XI–XII were incubated in HNK-1^{Au} at room temperature for 20 min and then at 37°C for 40 min, and washed extensively in Pannett-Compton saline. During this incubation period, cells expressing the HNK-1 epitope on their surface endocytose the antibody-gold complex and become labelled regardless of whether or not they continue to express the epitope during subsequent development. The posterior marginal zones of these labelled embryos were grafted into host embryos at the same stage of development and the operated embryos cultured for 8–16 h by New's (1955) method, until they had reached stages 3–4. The embryos were then fixed in buffered formol saline for 45 min to 1 h, embedded in paraffin wax and sectioned at 12 µm. After dewaxing in xylene and rehydration with a series of alcohols, the gold labelling was intensified by incubating each slide twice, 20 min each time, using IntenSE (Janssen) according to the manufacturer's instructions. They were then dehydrated through alcohols, cleared in xylene and

mounted in DePeX for examination either by bright-field microscopy or by confocal microscopy in reflection mode.

HNK-1/complement experiment

In a series of 10 experiments, donor embryos at stage XII were explanted and their posterior ends marked with carmine by touching the endoblast of the *area opaca* at the predicted posterior end with a tungsten needle that had been placed in a container of insoluble carmine powder. The embryos were then incubated for 20 min at room temperature with undiluted HNK-1 supernatant; an equal volume of guinea pig complement (Calbiochem) was then added and the embryos incubated for a further 1 h at 37°C and then washed extensively as described previously (Stern and Canning, 1990). Host embryos at the same stage of development were explanted from their eggs, their anteroposterior polarity marked with carmine as described above and their hypoblasts and the deep portion of their posterior marginal zones excised with fine tungsten needles. The deep portion of the posterior marginal zone (or, in a few cases, the full thickness of the posterior marginal zone) from a treated embryo was then grafted into the same position and orientation in a host. The graft was labelled with one or more spots of DiI (see above) to determine the extent of its contribution to the chimaeric embryo. The grafted embryos were allowed to develop in New (1955) culture to stages 3–4, fixed in buffered formol saline, photographed by bright-field and epifluorescence and processed for wax histology.

Four series of control experiments were carried out.

(a) In 5 embryos, the same operation as described above was done with an untreated donor. All of the operated embryos developed a normal hypoblast and a normal primitive streak, and the contribution of the graft was identical to that seen in unoperated embryos (see 'Fate mapping with DiI and DiO' in Results section).

(b) In 4 experiments, the donor embryos were treated with undiluted HNK-1 for 20 min, followed by addition of the same volume of Pannett-Compton saline and 1 h incubation. All developed normally and a normal hypoblast and primitive streak formed.

(c) In 4 operations, the donors were treated for 20 min with saline, followed by a 1 h incubation in a 1:1 mixture of saline and guinea pig complement. Three developed a normal hypoblast and a normal primitive streak axis, and the remaining embryo failed to develop.

(d) 8 stage XII embryos that had been treated with HNK-1 and complement, two treated with HNK-1 alone and three treated with complement alone were cultured. Only those treated with HNK-1 and complement failed to develop, as reported previously (Stern and Canning, 1990). This control experiment was done to test the effectiveness of the batches of antibody and guinea pig complement used in the present study.

Excision of portions of the marginal zone (Fig. 4)

In one series of experiments ($n=19$ embryos), the hypoblast and the entire thickness (including epiblast and deep components, as a ring about 200 µm in width) of the marginal zone were extirpated from stage XI–XII embryos in New (1955) culture, using fine steel needles made from A1 entomological pins. The outer *area opaca* was then replaced so that it contacted the cut edge of the inner *area pellucida* (Fig. 4A), because it has been shown that the presence of *area opaca* tissue improves the development of early blastoderms (Belairs *et al.* 1967). In these embryos, it was found to be important to absorb all remaining fluid from the region between the outer and inner regions to allow healing. This

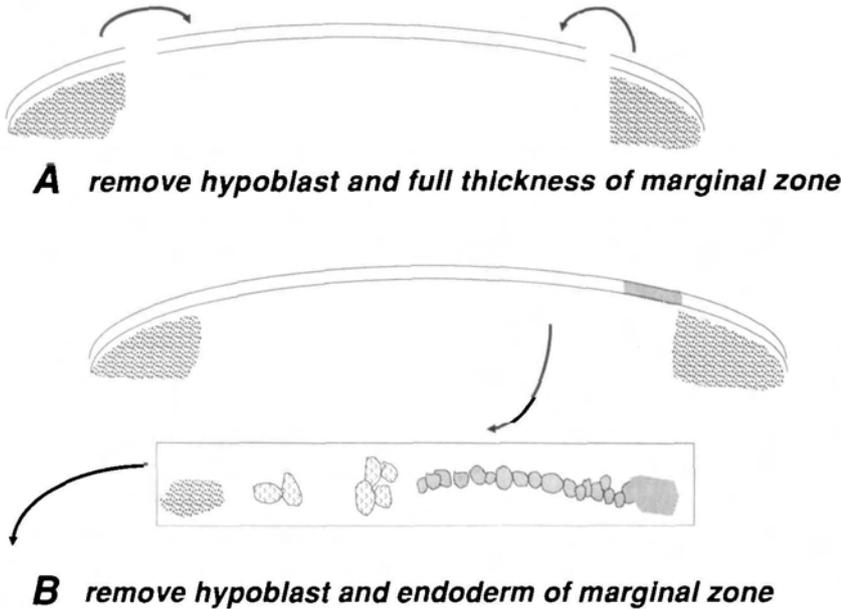


Fig. 4. Diagram of the margin excision experiments described in the text. Compare these with the diagrams of stage XII embryos in Fig. 1. In **A** the hypoblast and the whole thickness (including epiblast and deep layers) of the marginal zone is extirpated and the remaining portions of the *area opaca* brought into contact with the remaining part of the central *area pellucida* epiblast. In **B** the hypoblast and the deep portions of the marginal zone are extirpated. In both of these experiments, the region extirpated is a ring, extending all around the margin of the *area pellucida*.

was achieved by careful suction through a capillary tube pulled from a Pasteur pipette applied to the region of the graft, which also served to force together the inner and outer regions. The posterior region of the embryo was marked with carmine as described above prior to culture by New's (1955) technique. In three control embryos, the marginal zone was removed and replaced. All of these developed normally to stage 5. In another three embryos, the entire thickness of a submarginal portion of the *area opaca* was extirpated. Two of these embryos developed normally and the remaining embryo did not develop. In this embryo, the graft had failed to heal; a large gap filled with fluid had formed at the site of the operation.

In a second series of ablation experiments ($n=11$ embryos), the hypoblast and the deep component of the marginal zone of stage XI–XII embryos were removed using fine needles (Fig. 4B). A carmine mark was placed at the posterior end of each operated embryo, which was then cultured by New's (1955) technique to stages 3–5. A further two embryos were operated at stage XI as described above, but the hypoblast was left in place; both embryos were filmed using the time-lapse video techniques described above.

Results

Time-lapse video microscopy

High- and low-power time-lapse video films were made of the ventral surface of embryos developing in New (1955) culture from stage X. Fig. 5 gives examples of frames from these films. In general, the pattern of morphogenetic movements of the forming hypoblast confirmed those obtained from previous studies by time-lapse filming and carbon marking (Spratt and Haas, 1960a; Vakaet, 1970) (see Fig. 6 for summary of movements). Two regions were studied in most detail: the movement of 'islands' of primary hypoblast cells present at stage X and the anterior-directed movement of cells derived from the posterior marginal zone.

Each island of cells seen at stage X contains about 5–20 cells (although two or more islands may be in

contact with each other); there are about 30–200 such islands scattered over the ventral surface of the blastoderm, with many single cells intervening between them. The range of cell diameters in these islands is 25–200 μm (most are in the range of 30–50 μm). The larger cells tend to be found singly rather than forming part of islands, especially at the anterior end of the embryo (Fig. 5C). Although each cell within the island showed rotational motion about its own axis, little translocation either of the cells or of the islands was seen until the islands were met by a sheet of cells, migrating anteriorly from the posterior margin. This occurred at stage XI in the posterior third of the embryonic disc, at stage XII in the middle of the disc and at stage XIII in the anterior third; these stages are defined, in fact, by the position of the leading edge of the hypoblast sheet (Eyal-Giladi and Kochav, 1976).

The cells migrating from the posterior margin were somewhat smaller (average: 25 μm ; range: 20–40 μm) than those in the islands and moved at an average rate of 80 $\mu\text{m h}^{-1}$. The progress of the leading edge of the hypoblast cell sheet seemed to increase in speed from about 60 $\mu\text{m h}^{-1}$ at the start of migration (early-stage XI) to up to 250 $\mu\text{m h}^{-1}$ at mid-stage XII, and it slowed down again to about 70 $\mu\text{m h}^{-1}$ at the start of stage XIII. Much faster moving (generally 400–600 $\mu\text{m h}^{-1}$, but speeds of up to 1250 $\mu\text{m h}^{-1}$ were measured), smaller (5–15 μm) cells were also seen, particularly in posterior regions; these seemed to move in random, changing directions, between the islands of cells at stages XI and XII. It is likely, however, that they are also present at later stages, but the formation of the hypoblast as a coherent sheet of cells prevented them from being observed directly. These smaller cells appeared fibroblastic, in contrast with those migrating from the posterior margin or those in the islands, which were more spherical. They seemed to be associated closely with the epiblast and appeared to increase in number with development.

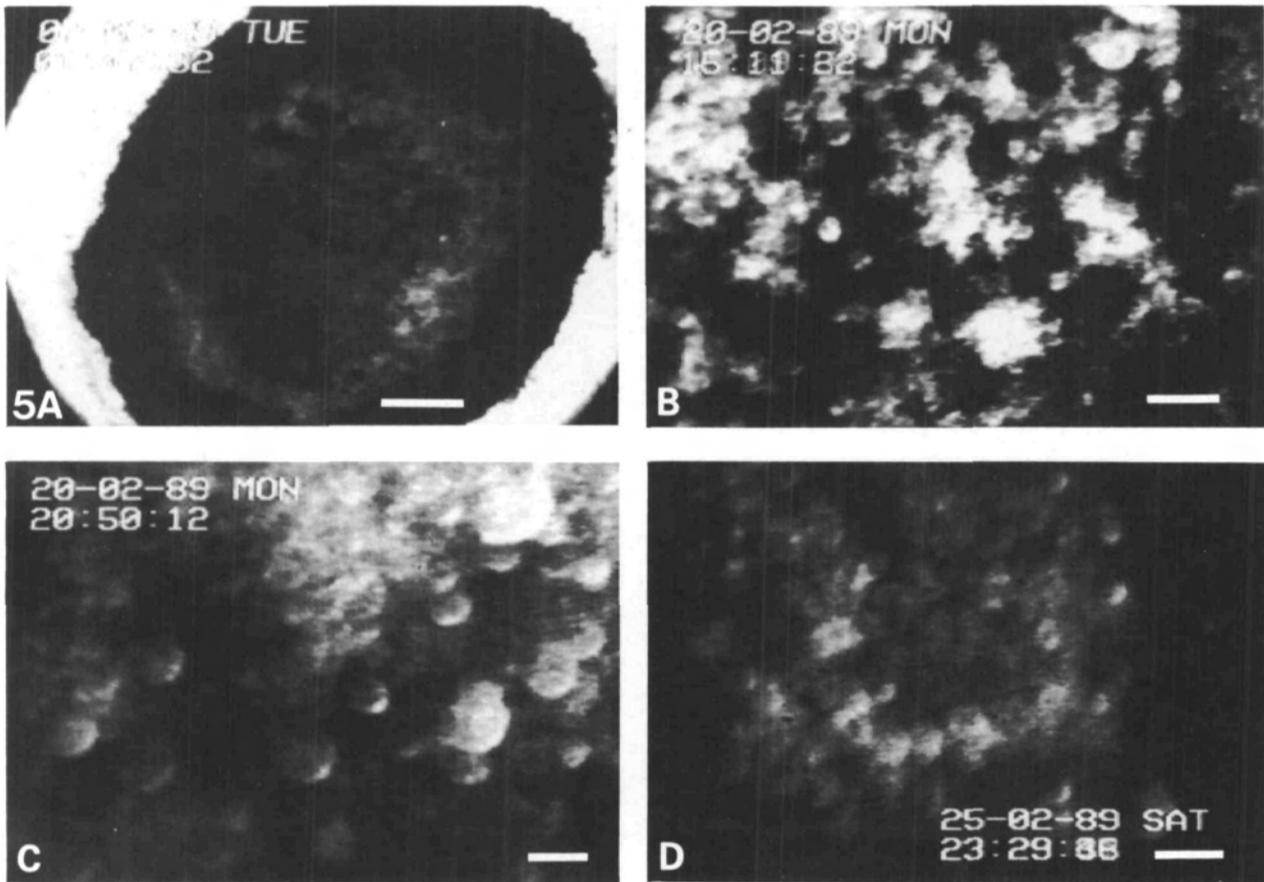


Fig. 5. Examples of frames from time-lapse films, taken through the video copy processor. (A) Low power view ($2.5\times$ objective) of a blastoderm at late-stage XII. The leading edge of the sheet of secondary hypoblast cells can be distinguished, parallel with the boundary between the *area opaca* (outer, dark) and *area pellucida* (inner, lighter). Posterior to the left. Transmitted light. Scale bar, $450\ \mu\text{m}$. (B) Intermediate magnification frame of a film of a stage XI embryo. In this reflected light view of the ventral surface, many islands of primary hypoblast can be seen scattered over the ventral surface of the embryo. Posterior to the upper left corner of frame. Scale bar, $200\ \mu\text{m}$. (C) Another frame from the same film as B above, taken at the anterior end of the embryo, which is now at late-stage XII. The leading edge of the secondary hypoblast sheet lies towards the upper left third of the frame, and many single, large, primary hypoblast cells can be seen just anterior (lower right) to it. Scale bar, $200\ \mu\text{m}$. (D) Higher magnification frame from a film of a stage XI embryo (now at stage XII), showing the dense sheet of secondary hypoblast cells after the primary hypoblast islands have been swept away from the field. Nomarski interference contrast optics. Posterior to the left. Scale bar, $50\ \mu\text{m}$.

Occasionally, violent funnel-like, 'focal' contractions were seen to occur in the epiblast as viewed from the ventral side at stages X–XII, similar to those seen in earlier time-lapse studies (Stern and Goodwin, 1977). These were more common towards the posterior pole of the *area pellucida*. The contractions were associated with the appearance of increased activity of the smaller, motile cells, but it was not possible to determine whether this increased activity reflected an associated increase in the number of these motile cells or an increase in their rate of movement.

Fate mapping with the carbocyanine dyes, DiI and DiO

The carbocyanine dyes, 1,1'-dioctadecyl-3,3',3'-tetramethyl indocarbocyanine perchlorate (DiI) and 3,3'-dioctadecyloxycarbocyanine perchlorate (DiO) were used to produce a fate map of the epiblast and

deep layers of the posterior marginal zone of the chick embryo at stages XI–XIII. In most embryos, one of the dyes was applied as a localized spot to the epiblast portion and the other as a localized spot to the endodermal portion at the same point of the circumference of the embryonic margin. Each spot of dye labelled some 10–50 cells. The results obtained with this technique are summarized in diagrammatic form in Fig. 6, and a few examples shown in Figs 7 and 8.

Labelled epiblast cells in the posterior marginal zone never contributed to the hypoblast. They generally became incorporated into the primitive streak and elongated with it in the anterior direction. Eventually, in those embryos that were allowed to develop sufficiently, labelled cells were found in the definitive endoderm and in the most dorsal (epiblast) portions of the streak (Fig. 7A,B,D; Fig. 8C–E). Labelled cells never contributed to the lateral mesoderm. Marks

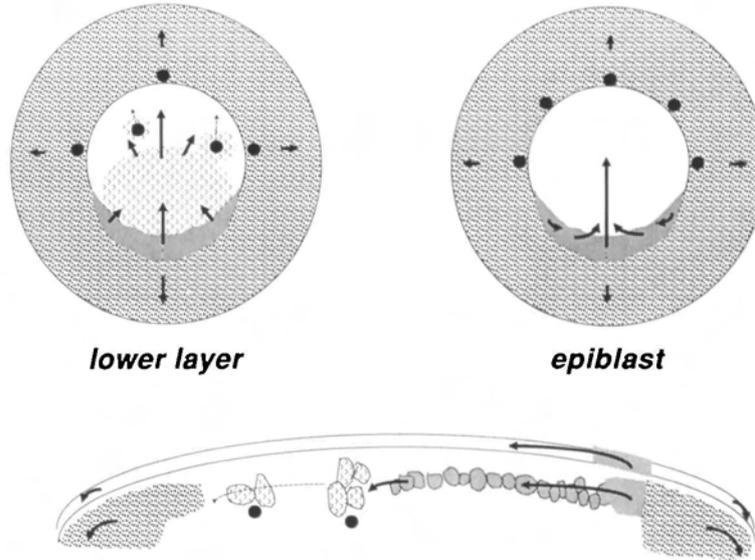


Fig. 6. Diagram summarising the morphogenetic movements of the deep (left) and superficial (right) portions of the marginal zone from stage XII, based on time-lapse films and fate mapping with carbocyanine dyes. The small black circles represent areas that do not appear to move actively (non-posterior margin and islands of primary hypoblast cells). The interrupted arrows denote regions of primary hypoblast which move only after being met by the expanding secondary hypoblast. In the section (below), anterior is to the left and posterior to the right (c.f. Fig. 1). Note that while the morphogenetic movements of the deep layer of the margin expand the secondary hypoblast in a centripetal way, the movements of the ectoderm portion of the posterior margin converge to a narrow region at the posterior pole of the *area pellucida*. Note also that in both layers movement of marginal cells towards the *area pellucida* is confined to the posterior end; in submarginal regions of the *area opaca* the movements of both layers are those of generalized expansion, and are centrifugal around the whole circumference.

placed in other marginal positions, away from the posterior pole of the embryonic disc, did not contribute cells that migrated into the centre of the blastodisc. Instead, a few cells moved circumferentially around the margin, most often towards the posterior pole.

Cells labelled in the deep portion of the posterior margin contributed many cells to the hypoblast. In embryos examined at or before stage 3, the labelled cells were seen to form a stream emanating from the point of labelling in a radial and anterior direction (in agreement with the movements of cells of this layer published previously; e.g. Spratt and Haas, 1960a; Vakaet, 1970). In older embryos, the labelled cells were often restricted to the anterior germinal crescent region, to which the hypoblast becomes confined (Fig. 7A,B,D). In almost every case, a group of labelled cells (about the same number as were originally labelled) was seen to remain at the site of labelling. Slightly different results were obtained from embryos labelled at stages XI–XII⁻ as compared to those labelled at stages XII⁺–XIII. In those labelled at the earlier stages, marked cells were found in the anterior germinal crescent at stage 4 (Fig. 7D). In those labelled later, the marked cells were confined to a region corresponding to the junctional endoblast, at the posterior end of the embryo (Fig. 8A,B). When cells in marginal regions other than the posterior end were labelled, their progeny were seen to migrate much less extensively than from the posterior margin; movements tended to be circumferential.

Marks placed in the epiblast or deep layers of the *area*

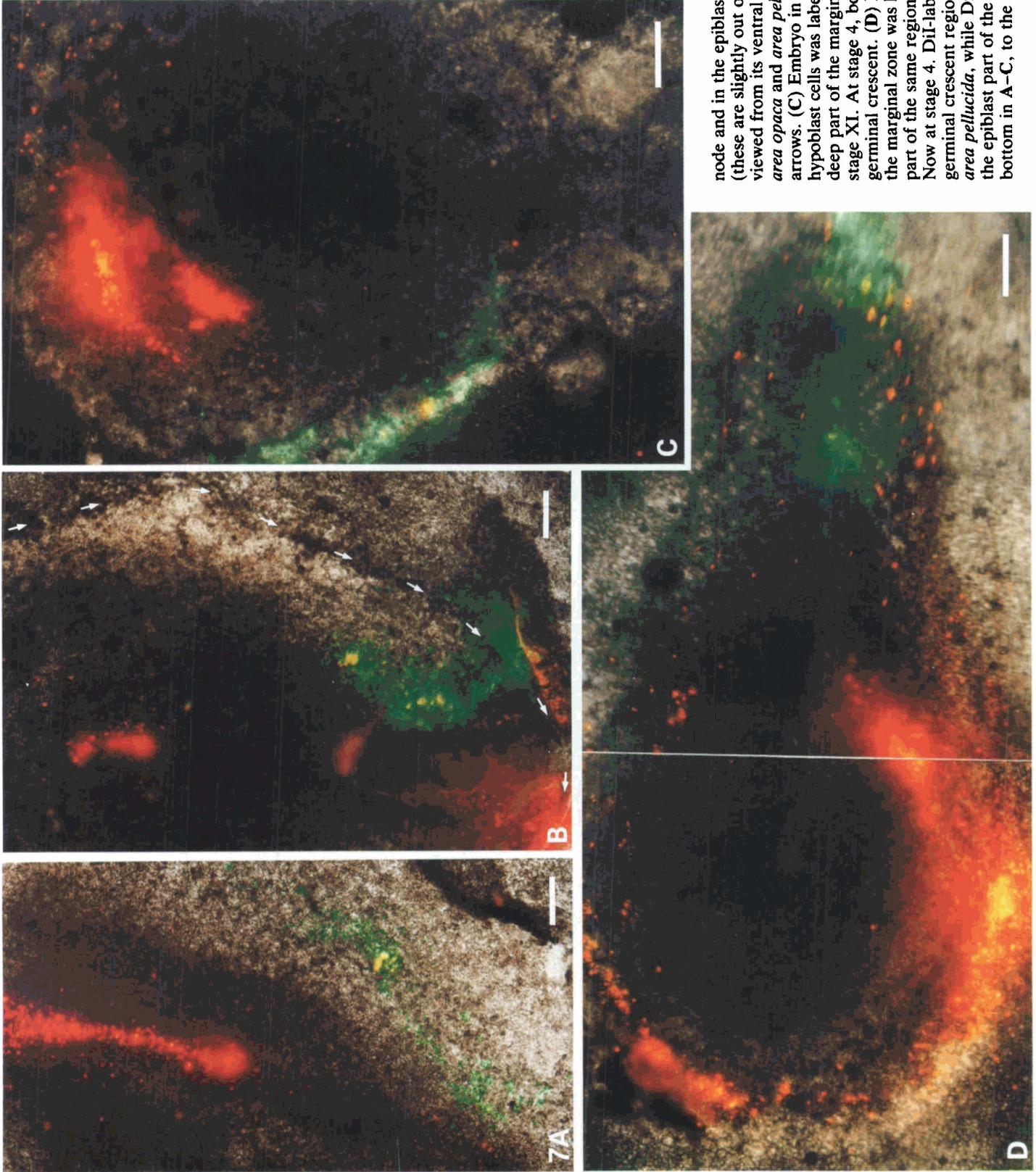
opaca further away from the centre of the blastoderm (submarginal) tended to produce cells that migrated centrifugally rather than centripetally. By mapping marks placed in the two concentric regions, the impression was obtained of a posterior marginal zone, from which cells migrate centripetally, which is about 100–200 μm wide.

In one series of 11 embryos at stage XI, one of the dyes was used to label a single island of primary hypoblast cells and the other dye to label a group of cells in the deep portion of the posterior margin, and the embryos allowed to develop in culture to stage 4–6. In all of these, cells labelled with each of the dyes were found in the anterior germinal crescent region (Fig. 7C).

Fate mapping of HNK-1-positive cells

In order to determine whether the HNK-1-positive cells found among the cells of the posterior marginal zone are the progenitors of the hypoblast (which is also HNK-1-positive; Canning and Stern, 1988), the technique described by Stern and Canning (1990) was used. Donor embryos ($n=7$) at stage XII were incubated in HNK-1 coupled directly to 15 nm colloidal gold (HNK-1^{Au}); during this period, the HNK-1-positive cells become labelled with HNK-1^{Au} and endocytose the antibody-gold complex. The embryos were then washed with saline and their posterior marginal zones used to replace the same region in similarly staged but unlabelled host embryos from which the entire hypoblast and posterior marginal zone had been extirpated

Fig. 7. Fate mapping with DiI and DiO. Triple-exposures (DiI with rhodamine optics [red], DiO with fluorescein optics [green] and bright-field on each frame) showing the distribution of DiI and DiO labelled cells. (A) Embryo in which the deep component of the posterior marginal zone was labelled with DiO and the ectodermal layer of the same region labelled with DiI at stage XI. The embryo was incubated to stage 4; the DiI labelled cells have contributed to a wedge shaped region of endoderm radiating anteriorly from Hensen's node, while the DiO labelled cells have contributed to the hypoblast of the *area pellucida*. (B) Embryo in which the deep component of the posterior marginal zone was labelled with DiO and the ectodermal portion with DiI at stage XI. At stage 4, DiO-labelled cells are found in the hypoblast of the *area pellucida* and DiI-labelled cells are found both in the endoderm around Hensen's node and in the epiblast portion of the primitive streak (these are slightly out of focus because the embryo is viewed from its ventral side). The boundary between *area opaca* and *area pellucida* is indicated by small arrows. (C) Embryo in which an island of primary hypoblast cells was labelled with DiI and cells in the deep part of the marginal zone labelled with DiO at stage XI. At stage 4, both markers are found in the germinal crescent. (D) Embryo in which the deep part of the marginal zone was labelled with DiI and the epiblast part of the same region labelled with DiO at stage XI. Now at stage 4, DiI-labelled cells are found in the germinal crescent region and around the periphery of the *area pellucida*, while DiO-labelled cells are restricted to the epiblast part of the primitive streak. Posterior to the bottom in A-C, to the right in D. Scale bars: 500 μ m.



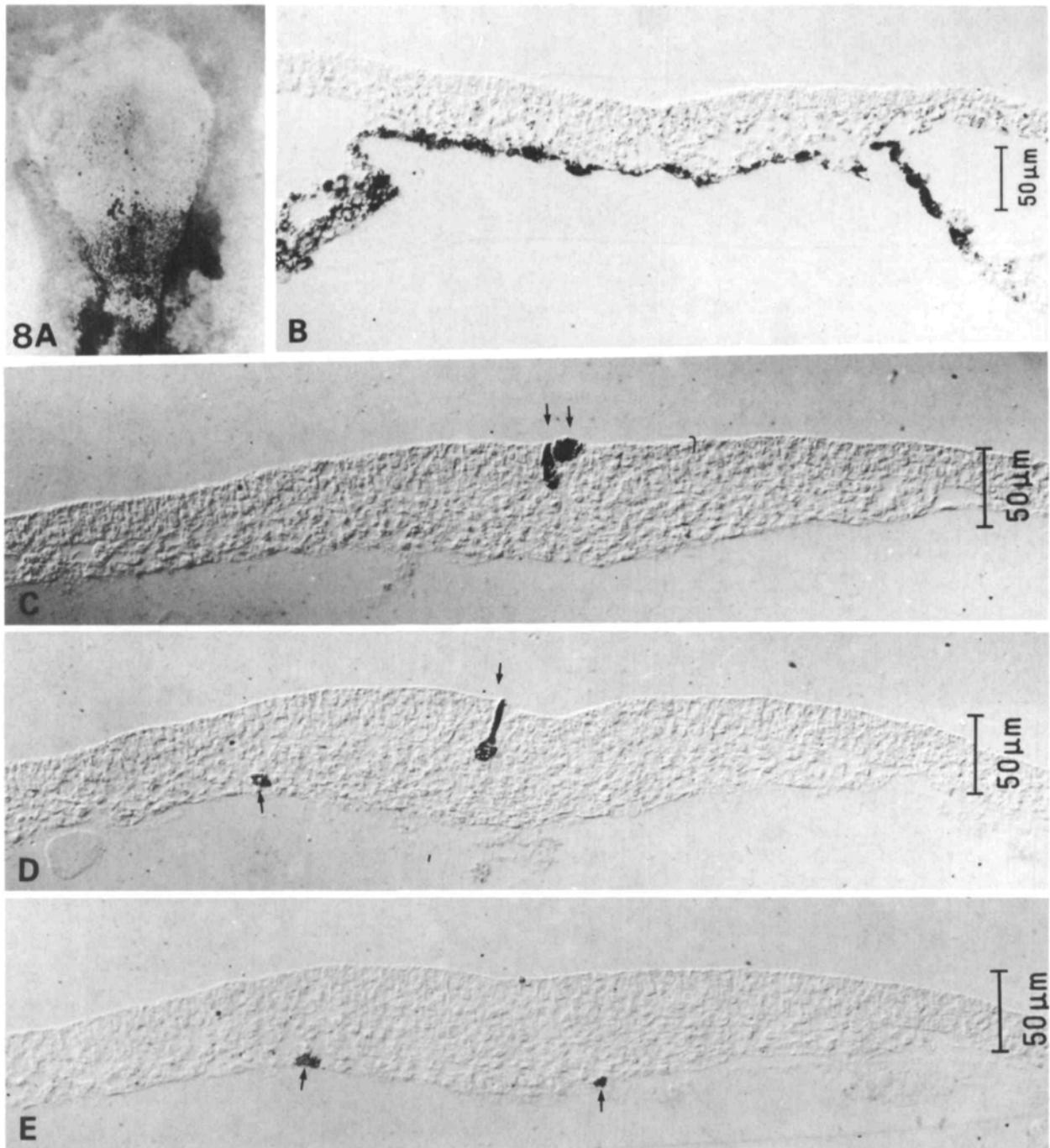


Fig. 8. Embryos labelled with DiI in the deep (A,B) or superficial (C,D,E) portions of the marginal zone, after photooxidation processing with DAB. Labelled cells appear black due to the DAB precipitate. (A) Embryo labelled with DiI applied at several large sites in the deep portion of the marginal zone at stage XII⁺, cultured until stage 4. Whole mount micrograph after photooxidation of the fluorescence, showing the extent of the contribution of the labelled marginal cells to the endodermal tissues of the *area pellucida*. (B) Transverse section through the same embryo as in A. Labelled cells are found exclusively within the lower layer, in a region corresponding to the junctional endoblast (see Fig. 1). (C–E) Transverse sections through an embryo that had been labelled with a single small (about 20 cells) spot of DiI in the superficial portion of the marginal zone at stage XI, cultured to stage 4. The pattern of labelling seen in this embryo was similar to that shown in Fig. 7A (DiI). Each of these sections contains two labelled cells. Posteriorly (C), labelled cells are restricted to the epiblast portion of the primitive streak itself. Near Hensen's node (D), cells are found both in the epiblast portion of the primitive streak and in association with the deep layer (definitive [gut] endoderm; cf. Fig. 1), while anterior to the primitive streak (E) labelled cells are restricted to the definitive endoderm. A, bright-field optics. B–E, Nomarski interference contrast optics.

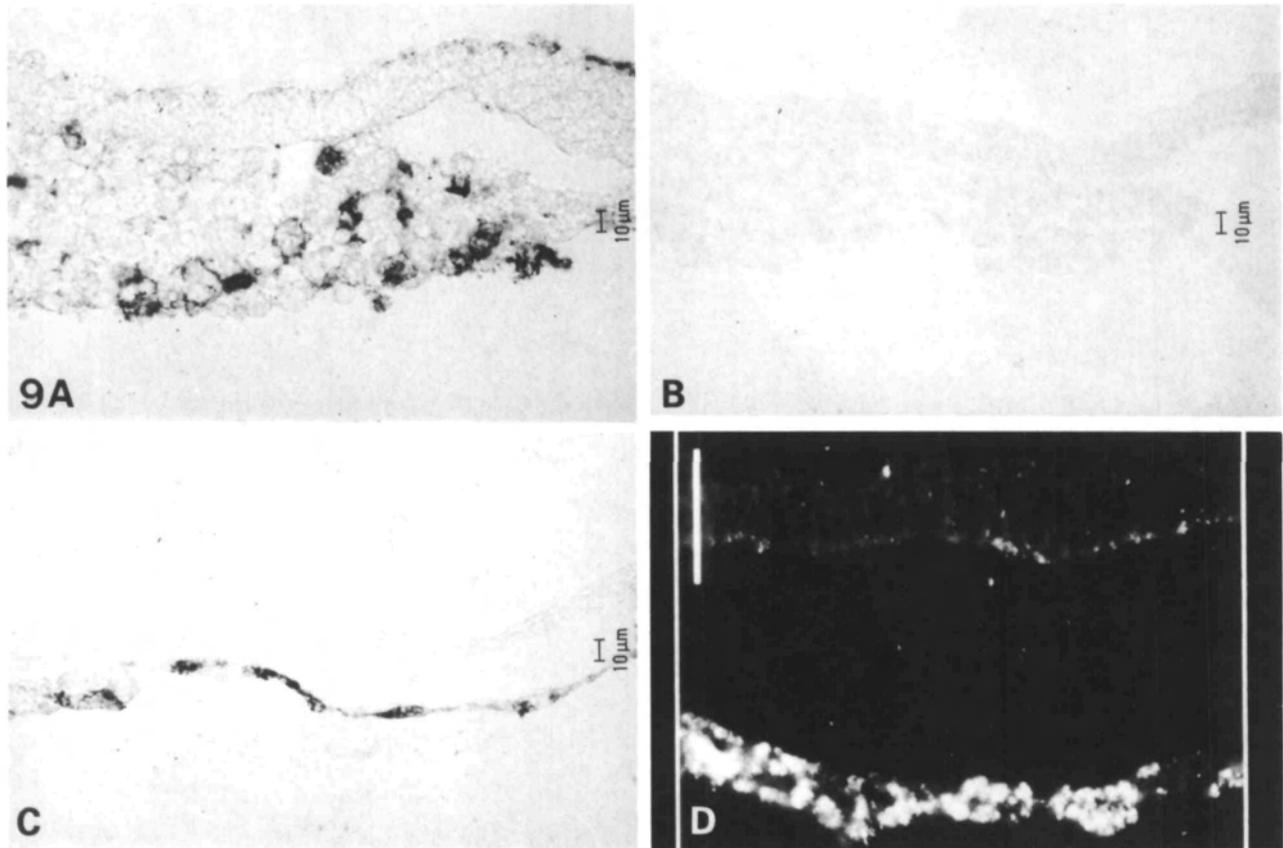


Fig. 9. Fate mapping with HNK-1^{Au}. (A) Transverse section through the posterior marginal zone of an embryo incubated in HNK-1^{Au} at stage XII, fixed immediately after incubation in the reagent. Some cells in the deep layer of the marginal zone are labelled, and have endocytosed the antibody-gold complex. Bright-field microscopy, after silver intensification; the silver grains appear dark. (B) Transverse section through the anterior marginal zone of the same embryo as in A. Cells are not labelled in this region, demonstrating the specificity of the HNK-1^{Au} reagent. (C) Transverse section through the anterior germinal crescent of an embryo (now stage 4) from which the hypoblast had been removed at stage XI, and the posterior marginal zone of which was replaced by a similar region from a HNK-1^{Au}-labelled donor embryo at the same stage of development. The grafted embryo was allowed to develop to stage 3⁺. The silver-intensified section shows gold-labelled cells exclusively in the hypoblast. Bright-field light microscopy; the silver grains appear dark. (D) Section through an embryo subjected to a similar treatment to that in C. Confocal microscopy in reflection mode, after subtraction of an image of the same section obtained by bright-field digital microscopy. The silver grains appear bright. Only the hypoblast shows intense labelling, but a few single grains are seen in the basal lamina of the epiblast, in agreement with earlier findings (Canning and Stern, 1988) in which this structure was found to be HNK-1-positive. Vertical bar, 50 µm.

($n=7$). After further incubation of the grafted embryo in New (1955) culture, cells derived from the grafted marginal zone can be visualized in paraffin sections by intensifying the gold labelling with silver (Janssen IntenSE).

The grafted embryos were allowed to develop for 8–16 h, by which time they had reached stages 3–4. In all cases, it was found that the hypoblast of the grafted embryos was composed of gold-labelled cells (Fig. 9C,D). Some gold-labelled cells were still found in the grafted marginal zone but not elsewhere in the embryo.

Two additional embryos that had been labelled with HNK-1^{Au} were fixed, sectioned and intensified immediately after incubation to examine the pattern of labelling. As expected, only some of the cells of the deep region of the posterior margin were labelled (Fig. 9A); the number of these labelled cells resembled the por-

tion of HNK-1-positive cells found in the margin by immunohistochemistry (Canning and Stern, 1988). The reagent had labelled cells deep in the marginal zone (Fig. 9A); penetration among the cells of this region was therefore not a problem. The specificity of the reagent was confirmed by comparing sections through the posterior (Fig. 9A) and other regions (Fig. 9B) of the marginal zone: all the labelled cells were located in the posterior region.

HNK-1/complement treatment

Of the 10 embryos which, after removal of the hypoblast and deep posterior marginal zone, had been grafted with deep posterior margins from HNK-1/complement treated donors, 6 developed a normal primitive streak but not a hypoblast (Fig. 10). Two embryos developed a primitive streak and a thin,

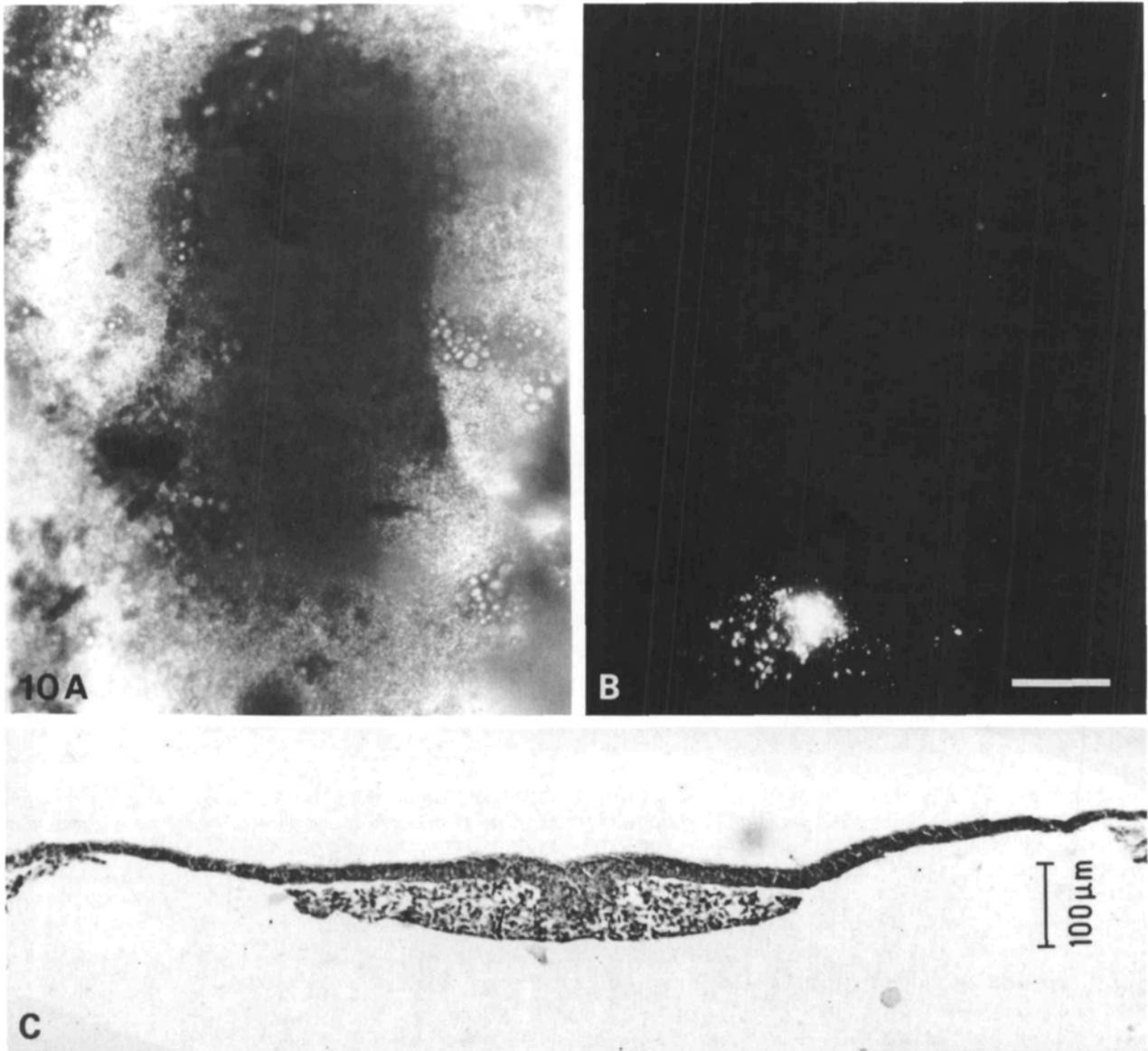


Fig. 10. Ablation of HNK-1-positive cells with HNK-1 and complement. (A) Whole mount of embryo from which the hypoblast and deep portion of the marginal zone had been removed at stage XI, and which had been grafted orthotopically with the deep part of a posterior marginal zone from a HNK-1/complement treated donor embryo at the same stage. The grafted embryo was cultured to stage 4. A primitive streak of normal appearance has formed. (B) Fluorescence image of the same embryo as in A, demonstrating that the cells of the grafted posterior margin, which had been labelled with DiI, remain confined to the region of the graft (posterior, bottom of photograph). Rhodamine optics; scale bar for A and B, 500 μm . (C) Transverse section through the primitive streak of the same embryo, stained with haematoxylin. The section confirms the presence of a normal primitive streak and the absence of the hypoblast.

abnormal hypoblast was present. The two remaining embryos did not develop normal primitive streaks; instead, a 'button'-like structure was present, protruding to the dorsal side of the embryo and containing mesoderm-like tissue. This structure resembled amphibian exogastrulae. In the 6 embryos in which no hypoblast had formed, the grafted tissue did not contribute cells to the *area pellucida* as judged by the movement of DiI-labelled cells. In those in which a thin, abnormal hypoblast was present, a few DiI-labelled cells were seen in the hypoblast.

Extirpation of the marginal zone

In 7 of the 11 embryos from which the hypoblast and the deep portion of the marginal zone had been removed, the hypoblast failed to regenerate but the primitive streak and other axial structures developed normally (Fig. 11A,C). The carmine mark placed at the predicted posterior end of the embryo corresponded to the posterior end of the primitive streak in each of these 7 embryos. In two of the remaining embryos, the primitive streak formed and a thin, abnormal hypoblast was present (this may have arisen from residual marginal

zone that had been spared at the time of excision; it is notable that in this embryo the position of the carmine mark agreed with the orientation of the primitive streak that formed). In another embryo, the primitive streak and hypoblast both failed to form, but an 'exogastrula' (see above) had formed. In the remaining embryo, no further development took place.

In a further two embryos, the deep portion of the marginal zone was removed at stage XI but the hypoblast was left in place. Both embryos were filmed during the post-operative culture period. It was found that the secondary hypoblast expanded by about 20% of its original surface area and then ceased to expand further. The primitive streak formed normally in both these embryos, and no movement of deep posterior marginal cells into the *area pellucida* was seen. Although both embryos were filmed only up to stage 4, they were allowed to develop in the incubator; they reached stages 7 and 8, respectively.

In 11 of the 19 embryos from which the hypoblast and the entire thickness of the marginal zone had been removed, the blastoderms expanded normally but did not produce a recognisable primitive streak (Fig. 11B). The morphology of the embryos was grossly altered, and the central *area pellucida* often appeared to be thickened. However, in histological sections, a structure resembling the primitive streak was seen in 9 of these embryos (Fig. 11D,E); the hypoblast was always absent in these embryos, but a layer similar to the normal definitive endoderm was present. In one of the remaining embryos, no expansion or further development took place and I assume that this embryo had died during the culture period. In four embryos, expansion of the blastoderm took place normally but the primitive streak failed to form; the hypoblast was also missing. In the remaining embryos, the graft had failed to heal properly and a large fluid-filled space was found between the outer and inner regions.

Discussion

The results obtained in this study can be summarized as follows (the experiments from which these conclusions are derived are shown in square brackets at the end of each conclusion):

(a) The deep (endodermal) region of the posterior marginal zone contributes cells to the secondary hypoblast and junctional endoblast in stage XI–XII embryos. When labelled early (stage XI–early XII) cells contribute to the secondary hypoblast; when labelled later (middle of stage XII–XIII), cells contribute mainly to the junctional endoblast [DiI/DiO labelling, HNK-1^{Au}, time-lapse video].

(b) The epiblast component of the posterior marginal zone contributes to the ectodermal portions of the primitive streak and to definitive endoderm derived from it [DiI/DiO labelling].

(c) Removal of the deep regions of the marginal zone at stage XI–XII prevents secondary hypoblast formation but does not prevent the formation of the primitive

streak. Removal of the entire thickness of the marginal zone (including epiblast and deep portions) prevents hypoblast formation and interferes with the elongation and normal morphology of the primitive streak, but it does not prevent the differentiation of primitive streak characteristics [extirpation experiments].

(d) Non-posterior regions of the marginal zone and submarginal cells show much less, if any, centripetal migration of cells from either layer into the *area pellucida* [DiI/DiO labelling, time-lapse video].

(e) The hypoblast cells derived from the deep portion of the posterior marginal zone ('secondary' hypoblast) are considerably more motile than those that are present from earlier stages of development (islands of 'primary' hypoblast). The latter hardly move at all until met by the migrating cells derived from the posterior margin. Both the primary and the secondary hypoblast cells end up in the anterior germinal crescent [time-lapse video, DiI/DiO labelling].

(f) HNK-1-positive cells in the posterior marginal zone contribute to the hypoblast. Ablation of the HNK-1-positive cells in this region often results in a failure of the secondary hypoblast to form but does not prevent the formation of a primitive streak [HNK-1^{Au}, HNK-1/complement].

These results show that the posterior marginal zone of the early chick embryo is a complex region, comprising at least two distinct cell types. Cells of the deep portion contribute to the hypoblast, and it seems likely that there is further heterogeneity among these cells, at least as revealed by immunoreactivity with antibody HNK-1 (Canning and Stern, 1988). The epiblast component, on the other hand, does not appear to contribute cells to the secondary hypoblast if labelled at stages XI–XII. Rather, most of the cells derived from posterior marginal zone epiblast become incorporated into the most superficial portions of the primitive streak. The only contribution of this region to the endodermal layer occurs later, when cells derived from it that have become incorporated into the primitive streak emerge from it as definitive (gut) endoderm. These results do not lend support to the assumption (e.g. Khaner *et al.* 1985) that it is the epiblast of the posterior marginal zone that is responsible for its contribution to the hypoblast and for its role in the formation of the primitive streak. Instead, the results indicate that the contribution to the hypoblast and the role in primitive streak formation are two separate properties, and that each of these properties resides in a different germ layer component of the posterior margin.

Origin of the hypoblast

The finding that the motility and appearance of the cells of the primary hypoblast differ from those of the cells of the secondary hypoblast indicate that the hypoblast is a more heterogeneous tissue than superficial examination of its histological appearance suggests. The primary hypoblast contains larger cells and is almost immotile, while the cells of the secondary hypoblast are of a consistent size and migrate actively over a large distance. The cells of the secondary hypoblast tend to form

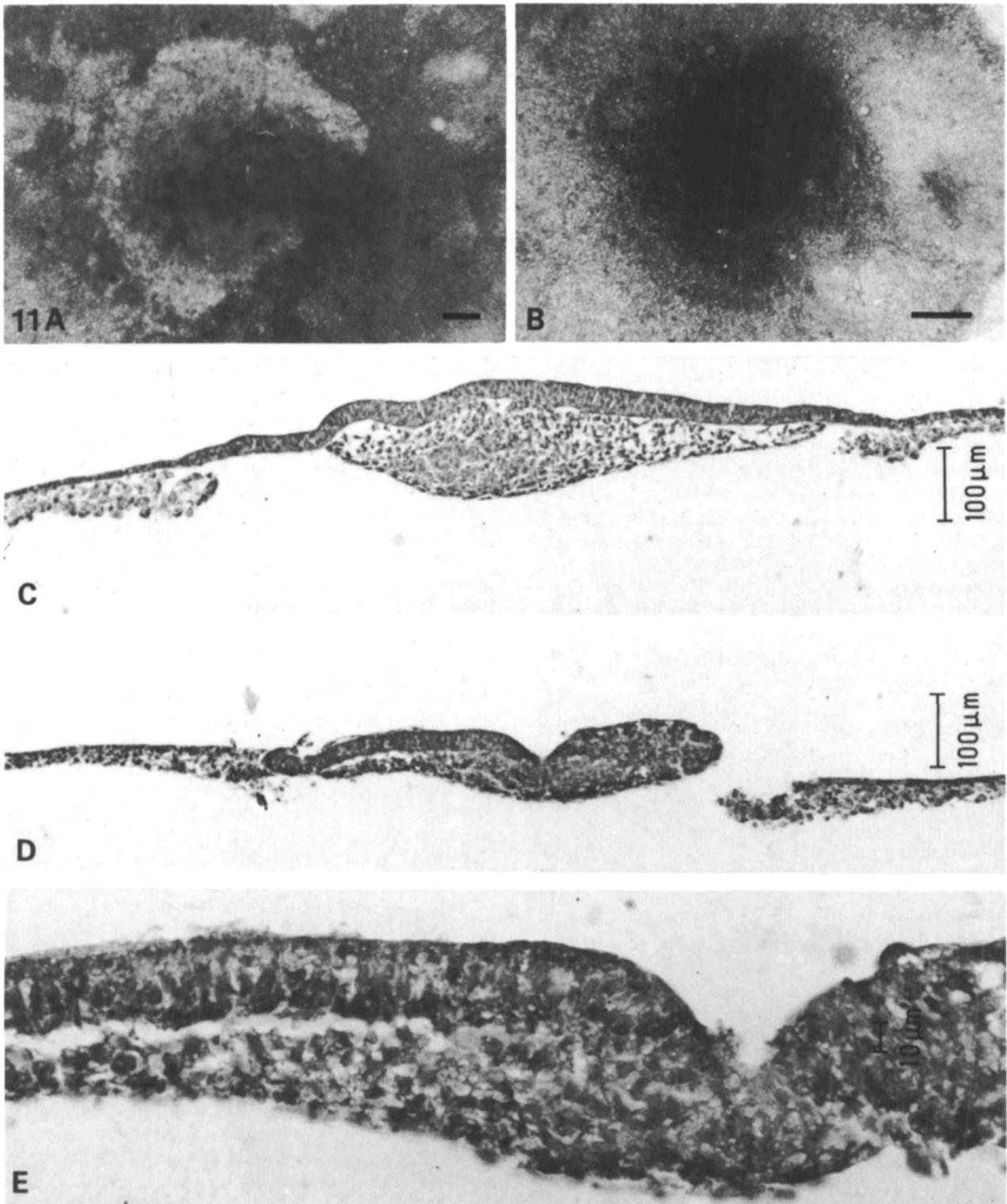


Fig. 11. Extirpation of the marginal zone. (A) Embryo from which the hypoblast and the deep portion of the marginal zone were removed at stage XI, and which was subsequently cultured to stage 3⁺. A primitive streak has formed, but no hypoblast is present. Posterior end to the right. Scale bar, 500 μm. (B) Embryo from which the hypoblast and the entire thickness (ectodermal and endodermal portions) of the marginal zone were removed at stage XI, and which was subsequently cultured for 16 h. The central *area pellucida* has a thick appearance, and no axial structures can be recognized in the whole mount. Scale bar, 500 μm. (C) Parasagittal section through embryo in A, stained with Harris's haematoxylin; posterior to the right. The section confirms the presence of a normal primitive streak and the absence of the hypoblast. (D) Section through embryo in B, stained with Harris's haematoxylin. The central *area pellucida* is thickened by a mass of mesoderm-like cells, grouped under a primitive-streak-like structure which could not be seen in the whole mount. (E) Higher power view of the primitive-streak-like structure in D.

a continuous sheet, while those of the primary hypoblast can only be found singly or in small groups. Time-lapse films showed that the primary hypoblast cells do not translocate until they are met by the rapidly migrating cells of the secondary hypoblast, derived from the posterior margin of the embryo. The films gave the impression that the primary hypoblast cells are forced forwards by the migrating secondary hypoblast.

Despite this heterogeneity of behaviour and morphology of the cells of the primary and secondary hypoblast, both cell populations are recognized by monoclonal antibody HNK-1. The primary hypoblast islands are thought to be derived from the centre of the *area pellucida* by ingression at stage IX–X, and they already express the HNK-1 epitope while in the epiblast at stage X (Canning and Stern, 1988; Canning, 1989). The secondary hypoblast also expresses the HNK-1 epitope, as do some cells in the endodermal part of the posterior (but not elsewhere) marginal zone (Canning and Stern, 1988). For this reason, it seemed plausible that the HNK-1-positive cells seen in the deep portions of the posterior marginal zone might be the progenitors of the secondary hypoblast. The present results support this hypothesis: cells labelled by HNK-1^{Au} contribute to the hypoblast, and ablation of HNK-1-positive cells with HNK-1 and complement interferes with the regeneration of a normal hypoblast.

The two embryos grafted with HNK-1/complement-treated marginal zones in which a thin hypoblast developed may be significant. One possibility is that some HNK-1-positive cells survived the HNK-1/complement treatment, which receives support from the finding that the graft did contribute some DiI labelled cells to the regenerated hypoblast in these embryos. However, the possibility that HNK-1-positive cells are not the **only** cells in the posterior margin that can contribute to the hypoblast cannot be excluded at present. A definitive answer to this question will have to await further experiments.

The present study shows, nevertheless, that the secondary hypoblast is derived exclusively from the **deep** portions of the posterior marginal zone, and that the epiblast does not contribute cells to this layer at stages XI–XII. This is not surprising, since the morphology of cells in the deep portions of the marginal zone (germ wall) resembles the morphology of hypoblast cells much more closely than does the epiblast of this region (see Bancroft and Bellairs, 1974; Bellairs *et al.* 1981; Stern and Ireland, 1981; Al-Nassar and Bellairs, 1982). This study also shows that the deep portion of the posterior marginal zone contributes cells to the junctional endoblast component of the lower layer after stage XII. The spreading of the sheet of hypoblast is therefore due, initially, to secondary hypoblast cells derived from the marginal zone, joined later by junctional endoblast cells derived from the same region. The two components of the endoblast of the embryo differ in the position at which they are found at later stages of development: the secondary hypoblast joins the islands of primary hypoblast at the anterior germi-

nal crescent, while the junctional endoblast forms the posterior part of the endoblast of the *area pellucida*.

The role of the posterior marginal zone in the formation of the primitive streak

The cells of the epiblast portion of the posterior marginal zone have a different fate from those of the deep part: they contribute almost entirely to the superficial (ectodermal) tissue of the primitive streak. It is perhaps interesting that carbocyanine dye labelled cells were never seen in mesodermal tissues by stage 4 after marking cells in the posterior marginal region of the epiblast. This finding suggests that, although cells derived from the epiblast of the posterior margin delineate the ectodermal portion of the primitive streak in some way, the mesodermal cells of the interior of the streak are derived from elsewhere. This is in agreement with recent experiments, which demonstrate that the epiblast of the whole of the *area pellucida* contains a mixture of cells, as recognized by antibody HNK-1: the positive cells in this mixture are the progenitors of the primitive streak mesoderm and endoderm, but do not contribute to the epiblast (Stern and Canning, 1990).

It therefore seems likely that the cells of the primitive streak are derived from two sources: the epiblast of the posterior marginal zone plays a role in marking the site at which mesendodermal cells derived from elsewhere in the embryo will collect to form a primitive streak, while itself contributing to the ectodermal portion of the streak and to some definitive endoderm. This conclusion provides an explanation for the finding that rotation of the marginal zone results in reversal of the anteroposterior axis of the embryo (Azar and Eyal-Giladi, 1981). It also provides a cellular basis for the gradient in 'embryo-forming potency' that has been reported to exist in the marginal zone (Spratt and Haas, 1960*b,c*; Khaner and Eyal-Giladi, 1989).

Further support for this conclusion is provided by the finding that removal of the deep layer of the marginal zone allows normal development of a primitive streak despite the failure of the hypoblast to regenerate, while removal of both the epiblast and deep portions of the marginal zone do not allow the development of a morphologically normal axis. This experiment suggests that the epiblast portion of the posterior margin is required for primitive streak elongation or for controlling the shape of the streak, independently of whether the secondary hypoblast is present or not and independently of the differentiation of primitive streak characteristics. We can therefore distinguish three separate aspects of primitive streak formation: (a) the differentiation of mesendodermal cells, which does not depend on the marginal zone, (b) the position of the primitive streak, which appears to depend on the epiblast portion of the posterior marginal zone but can perhaps be influenced by the hypoblast (Waddington, 1933), and (c) the shape and elongation of the streak, which also requires the epiblast portion of the marginal zone.

However, it should be pointed out that the finding that the central *area pellucida* is capable of producing a primitive-streak-like structure in the absence of the

entire thickness of the marginal zone appears to conflict with some of the conclusions of Eyal-Giladi and colleagues (e.g. Azar and Eyal-Giladi, 1979), who did not obtain development of primitive streaks in the absence of a marginal zone. At present it is impossible to understand the reasons for the difference between our results. However, the present results do not conflict with those of Khaner and Eyal-Giladi (1989) or those of Eyal-Giladi and Khaner (1989), but their results have been interpreted in terms of 'induction' by the posterior marginal zone. The present experiments neither support nor conflict with an inductive function of the marginal zone in the formation of the primitive streak. To distinguish between the two possibilities it will be necessary to combine their homoplastic and heteroplastic transplantations with cell marking experiments such as those presented in this paper.

It is more difficult, however, to account for the result of Waddington's (1933) and Azar and Eyal-Giladi's (1981) hypoblast rotation experiments, in which antero-posterior rotation of the hypoblast resulted in reversal of anteroposterior polarity of the primitive streak. There are three simple interpretations of these findings: (a) the hypoblast itself 'induces' the posterior marginal zone epiblast in terms of its ability to produce the epiblast portions of the primitive streak, (b) when the hypoblast is excised for rotation, the explant includes some associated mesodermal cells which may have already started to collect at the posterior end of the embryo and which might encourage more mesodermal cells to collect around them, or (c) the marginal zone serves as a source of a chemoattractant for presumptive mesoderm cells and the hypoblast is the vehicle through which it spreads.

The first of these interpretations has been favoured by Eyal-Giladi and her colleagues. However, extirpation of the hypoblast and posterior marginal zone endoblast at a much earlier stage (XI–XII) than that at which the hypoblast rotation experiments were done (XIII) allows normal development of a primitive streak in the absence of a hypoblast (our excision and HNK-1/complement experiments), suggesting that none of the endodermal portions of the embryo are required for the formation of a normal primitive streak.

In favour of the second hypothesis is the finding, in the present experiments, of small, highly motile, fibroblastic cells present at a very early stage of development particularly in posterior regions of the *area pellucida* (time-lapse filming). These might be early mesodermal cells that have started to ingress into the interior of the embryo at a much earlier stage (XI–XII) than their coalescence into the primitive streak (stage XIV-2). Moreover, it has been shown that hypoblast explants from stage XII–XIV embryos contain small fibroblastic cells that resemble mesoderm *in vitro* (Stern and Ireland, 1981). In conclusion, therefore, the possibility that rotation of the hypoblast in Waddington's and Azar and Eyal-Giladi's experiments included some mesodermal cells associated with the rotated hypoblast cannot be excluded.

The final possibility seems attractive because we have

shown that the mesoderm and some of the endoderm cells of the primitive streak are derived from a different source than those of the ectodermal portion of this structure (Stern and Canning, 1990 and this study). If the presumptive mesoderm cells are scattered throughout the *area pellucida*, they must be able somehow to find the site at which they will collect to form the streak. This suggestion can be tested by experiments; for example, it might be possible to use a collagen gel assay for chemotaxis towards the posterior marginal zone such as those used previously to study the outgrowth of neurons from the trigeminal ganglion (Lumsden and Davies, 1983; 1986). Experiments to distinguish between these possibilities are in progress.

Whatever the interpretation given to the hypoblast rotation experiments, cells of the epiblast portion of the posterior marginal zone must be different from cells of the epiblast of other regions of the embryo. This prediction is borne out by immunological experiments (Stern, Buckingham, Canning, Carlson and Maughan, in preparation), in which it is shown that the posterior marginal epiblast expresses a series of markers which single it out from the rest of the epiblast. It is therefore likely that cell heterogeneity exists within the epiblast from very early stages in its development.

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