Development 116, 819-830 (1992)
Printed in Great Britain © The Company of Biologists Limited 1992

The posterior section of the chick's area pellucida and its involvement in hypoblast and primitive streak formation

HEFZIBAH EYAL-GILADI, ANAT DEBBY and NOA HAREL

Department of Cell and Animal Biology, Hebrew University of Jerusalem, 91904 Jerusalem, Israel

Summary

Posterior marginal zone sections with or without Koller's sickle were cut out of stage X, XI and XII E.G&K blastoderms, labelled with the fluorescent dye rhodamine-dextran-lysine (RDL) and returned to their original location. In control experiments, a similar lateral section of the marginal zone was identically treated. Different blastoderms were incubated at 37°C for different periods and were fixed after reaching stages from XII E.G&K to 4 H&H. The conclusions drawn from the analysis of the distribution pattern of the labelled cells in the serially sectioned blastoderms concern the cellular contributions to both the forming hypoblast and the forming primitive streak. Koller's sickle and the marginal zone behind it were found to contribute all the centrally located cells of the growing hypoblast. The lengthening pregastrulation PS (until stage 3+ H&H) was found to be entirely composed of epiblastic cells that at stage X were located in a narrow strip anterior to Koller's sickle. A model is proposed to integrate the results spatially and temporally.

Key words: chick, marginal zone, hypoblast, primitive streak, cell movements.

Introduction

The mode of formation, the cellular composition and the developmental significance of the early chick's hypoblast have been the subject of severe dispute since the 1860s. The first theory, by now completely ruled out, was the delamination theory (see Pasteels, 1945), which stated that the two-layered blastoderm (stage XIII) arises by the appearance of a cleft, in the middle of the mass of cleavage cells, which gradually separates it into an upper and lower layer. The second theory supported the idea of an inrolling of a lower layer at the posterior border of the embryo (Duval 1884, 1888; Patterson, 1909). A third theory added the aspect of polyinvagination (or polyingression) of single or small groups of cells from the lower surface of the single-layered unincubated (stage X E.G&K) blastoderm (His, 1868; Nowack, 1902; Merbach, 1936).

A more recent approach supported the idea that the hypoblast was formed of two different cellular components: the polyingressing cells and the cells from a posterior source, which migrated anteriorly as a coherent sheeet. Both components were supposed to merge and form a lower layer in which they were morphologically indistinguishable (Spratt and Haas, 1960, 1965; Vakaet, 1962; Eyal-Giladi and Kochav, 1976). However, the cells of posterior origin were supposed by us to be functionally different (Azar and Eyal-Giladi, 1979; Khaner and Eyal-Giladi, 1986, 1989; Eyal-Giladi, 1984, 1991, 1992) and were believed to be the only ones capable of inducing a primitive streak (PS) in the epiblast. We therefore assumed that those cells occupy a very defined area within the hypoblast, underneath the forming primitive streak. As the cells of posterior origin are morphologically indistinguishable from the polyinvaginating cells there was also some disagreement concerning their exact origin. Spratt and Haas (1960, 1965), Eyal-Giladi and Kochav (1976), Azar and Eyal-Giladi (1979), Kochav et al. (1980), Khaner and Eyal-Giladi (1986, 1989) and Eyal-Giladi and Khaner (1989) predicted that the above posterior cells are derived from the posterior marginal zone (PM), which is part of the peripheral belt of the area pellucida (AP), and that they move into the hypoblast via Koller's sickle (KS). Stern and Ireland (1981) and Stern (1990) claimed that the posterior cellular contribution was derived from the germ wall margin (GWM), or as they called it "the deep portion of the marginal zone". All these conflicting ideas were probably partly due to the fact that most of the above cited papers have based their conclusions on observation of live material, conventional histological sections, or on indirect experimentation. In addition, there was also a terminology problem; different investigators using the same terms were referring to different things. This last and easiest problem has been dealt with recently by the adoption of a concensus terminology by a group of investigators of the early chick (Bellairs et al., personal communication). In the present study, we tried to elucidate the distribution pattern of posterior cells by vitally labelling the

PM and later following the behavior of its cells during incubation, from stage XII E.G&K and until stage 4 H&H (full PS).

Material and methods

Blastoderms of stages X, XI and XII E.G&K (Eyal-Giladi and Kochav, 1976; Kochav et al., 1980) were put in a Petri dish containing Ringer's solution and cleaned of adhering yolk (Fig 1A). The germ wall margin (GWM) (see also Fig. 8), which is a centrally directed extension of the deep part of the area opaca (AO), has been removed in most experiments to expose the MZ and its anterior border, KS (Fig. 1B). A posterior section of the blastoderm, including the AO and the PM, which in most cases also included the KS and a strip of the AP anterior to KS, was cut out (Fig. 1C). At stage X, a larger epiblastic strip anterior to KS was usually included while at stage XI, when KS is more clearly demarcated, the anterior strip was usually very narrow. In other blastoderms, the fragment was cut posterior to KS and the latter remained attached to the AP in front of it (Fig. 1D). In several experiments, the GWM was not removed and was included in the labelled fragment.

In a group of control experiments, a lateral section of the blastoderm was cut out and treated similarly (Fig. 1E). The right side of each fragment was marked with carmine and the left with carbon to mark the original orientation. The fragment was then transferred into an Ependorff test tube which contained a solution of 10.6 mM rhodamine-dextran-lysine (RDL) in Ringer's The fragment was incubated at 37°C for 35-55 minutes, conditions that were found in preliminary experiments to be optimal for both the labelling and a good survival of the tissue. After the incubation, the fragment was washed three times in clean Ringer's solution, each wash for 5 minutes. The fragment was then put back in the right orientation into its original place in the blastoderm, which was in the meantime transferred onto a vitelline membrane stretched on a glass ring, and put on a drop of solid albumin in a watch glass. After sucking away the excess fluid, the reconstructed blastoderm was incubated at 37°C until it reached one of the desired stage (XII, XIII E.G&K and 2,3,4 H&H). The blastoderms were then fixed for 2 hours in a modified Krotoski's et al. (1988) fixative, which contained 4% paraformaldehyde in 0.1 M PBS, pH 7.4. The fixed blastoderms were rinsed twice for 10 minutes in 0.1 M PBS and then kept in 70% ethanol before embedding in paraffin. The material was transversally serially sectioned at 15 μm and mounted on slides coated with gelatin. After deparaffinization, a few drops of Entellan and a coverslip were applied. The sections were viewed with a fluorescence microscope and every fifth section was drawn on a millimeter paper. An individual reconstruction was made from the drawings for every blastoderm. In the experimental group are included only blastoderms in which, at the time of fixation, either the hypoblast (stages XII-XIV E.G&K) or the PS stages 2-4 H&H (Hamburger and Hamilton, 1951) were clearly seen to develop in front of the labelled fragment. The blastoderms in which the labelled fragment was found to occupy a lateral position in relation to the developing PS, were added to the control group in which a lateral section of the blastoderm was originally labelled. The blastoderms were photographed in toto under the dissecting microscope on a dark background and with normal epi-illumination. The sections were viewed and photographed with a fluorescence microscope.

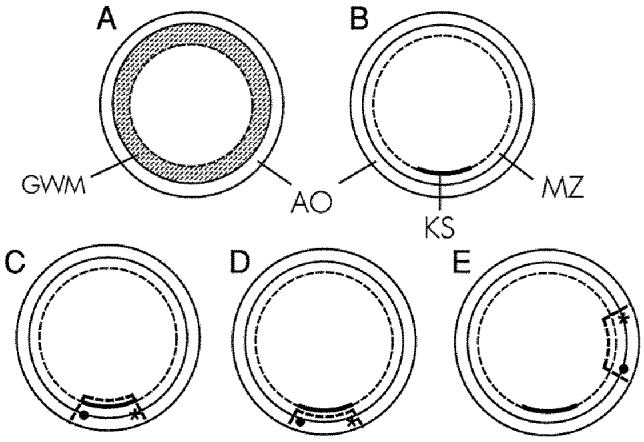

Fig. 1. (A) Ventral view of a stage X E.G&K blastoderm after its removal from the yolk. (B) Ventral view of the same blastoderm after removal of the germ wall margin (GWM) and exposure of the marginal zone (MZ). (C) Type of group A operation. The posterior marginal zone fragment includes Koller's sickle (KS) and a strip of epiblast anterior to it. (D) Type of group B operation. The posterior marginal zone fragment does not include KS and the epiblastic strip anterior to it. (E) A control experiment. The labelled fragment is of lateral marginal zone. Black spot, carbon mark; starlet, carmine mark; AO, area opaca.

Results

The results of the experimental group are divided into four subgroups according to the stage at which the development of the operated blastoderm was stopped and the blastoderm fixed.

Group A - *Experiments at stage X E.G&K (Fig. 2)*

A1 *(three blastoderms)*

The blastoderms were operated on at stage X (Fig. 3A) and fixed at stages XII or XIII (Fig. 3B). The examination of the serial sections revealed that, at both stages, the RDL-labelled cells occupy a relatively narrow longitudinal-median strip in the lower layer (Fig. 3C), which is posteriorly continuous with the initially labelled implant. There was no extension of RDL-labelled cells into the epiblast beyond the borders of the original implant at either of the two stages (Fig. 2, column 1A).

A2 *(two blastoderms)*

The blastoderms were operated at stage X and fixed at stage 2 H&H, when they already had a short PS. At stage 2 (Fig. 2, column 2A), there was no sign yet of a continuous mesoblastic layer between the epiblast and lower layer. The distribution pattern of RDL-labelled cells in both blastoderms was as follows. There was a remarkable expansion of labelled cells within the lower layer as compared to stage XII. The labelled cells occupied a continuous relatively big central area connected posteriorly to the original implant area and surrounded laterally and anteriorly by a belt of non-labelled cells. The main difference was found, however, in the epiblast, where the entire initial thickening of the forming PS was composed of labelled cells. All the other cells of the epiblast on both sides of the PS and anterior to it were not labelled.

A3 *(three blastoderms)*

The blastoderms were operated on at stages X-X+ and fixed at stage 3.

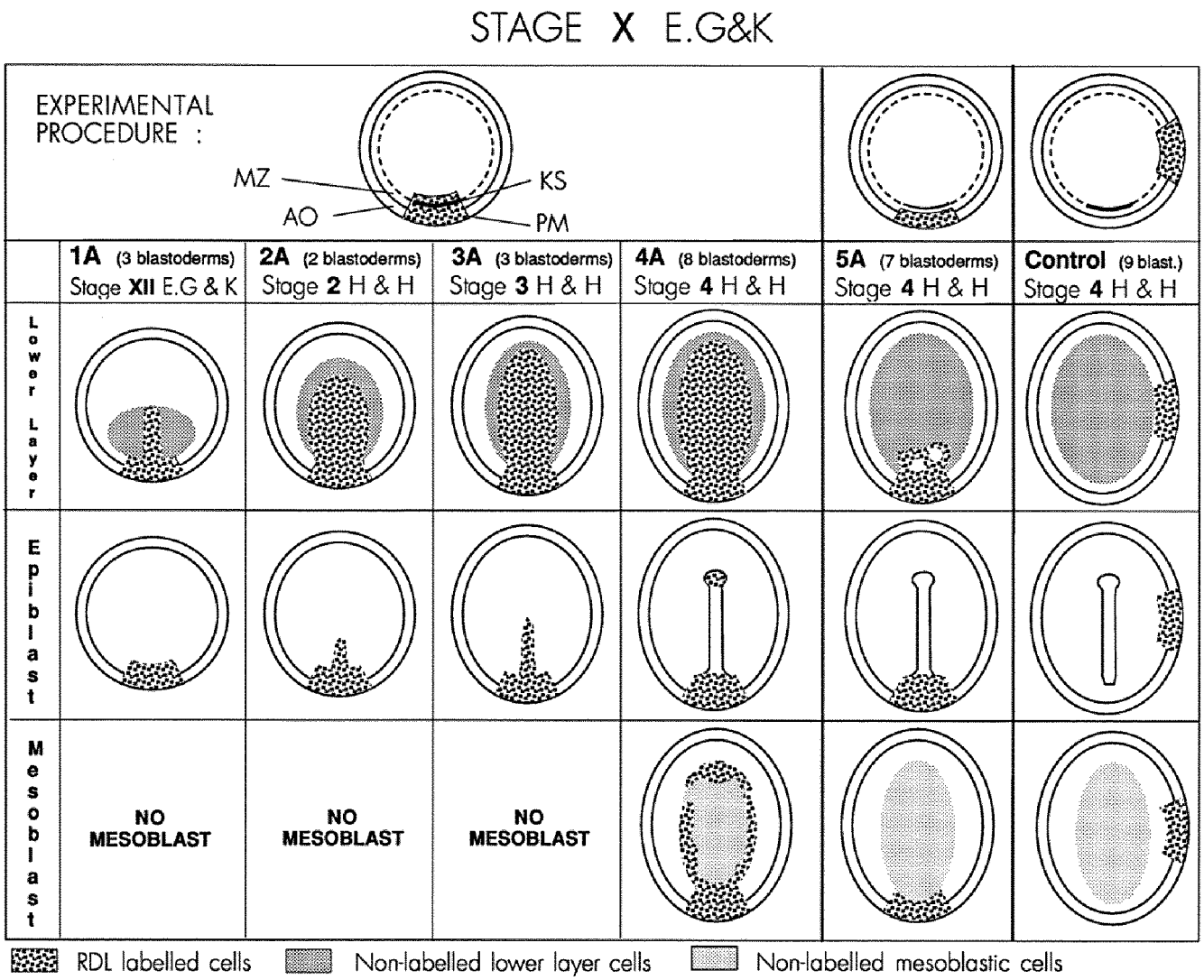

Fig. 2. A reconstruction based on the serial sections of blastoderms operated at stage X and fixed at different stages. The position of labelled cells is shown separately for each germ layer. AO, area opaca; KS, Koller's sickle; MZ, marginal zone; PM, posterior marginal zone.

In one blastoderm (no. 1), the PS developed exactly in front of the center of the labelled implant (Fig. 3D). In the sections (Fig. 3E) only two germ layers were observed, as the mesoblast has not yet formed as a layer. In the lower layer, the territory occupied by the RDL-labelled cells had expanded both anteriorly and laterally as compared to stage 2, its shape however remained very similar to the latter. Also in the epiblast the same pattern was found as in stage 2, namely, the PS alone was entirely composed of lablled cells, while the epiblastic cells surrounding it were not labelled (Fig. 3E; Fig. 2, column 3A).

The other two blastoderms are interesting in their own merit. First, because the operation was performed at stage X+, and, second, because the PSs did not develop in front of the center of the implants but occupied different, very instructive positions.

In one case in which the PS was seen to develop in front of the right border of the implant, the labelled territory in the lower layer was asymmetric, stretching underneath the PS and to the left of it. Anteriorly, it did not extend further than the PS itself. Also, the PS was only partially labelled (not as in blastoderm no. 1). In the other case the carbon clump that was intended to mark the left side of the implant was too big and formed a mechanical obstacle which locally prevented rapid healing (all the other edges healed normally). As a result, two independent developmental centers arose: one from the RDL-labelled marginal zone fragment and another from the intact non-labelled marginal zone lateral to the clump. The blastoderm that was fixed at stage 3 H&H had two parallel PSs. The serial sections demonstrated that the lower layer underneath the right PS was labelled while the lower layer underneath the left PS was not. Similarly the posterior section of the right PS was intensely labelled while the parallel section of the left PS was not. More anteriorly, labelled cells were found in both PSs, that seemed to fuse.

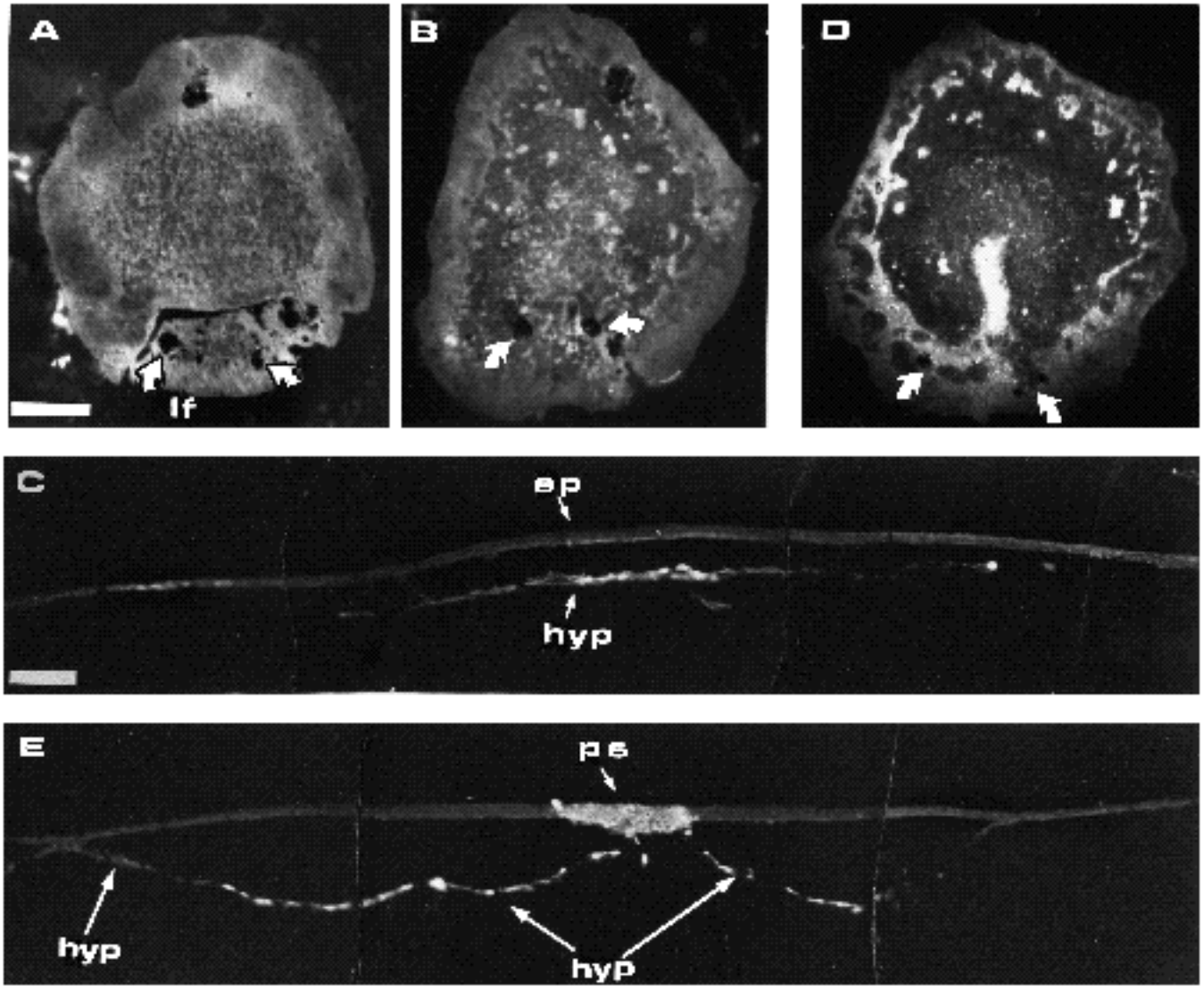

Fig. 3. (A) Operation at stage X. The labelled fragment (lf) included the KS and an epiblastic strip anterior to it. (B) Same blastoderm after incubation to stage XIII. The position of the healed fragment can be easily traced by the carbon (left) and carmine (right) spots indicated by the curved white arrows. (C) A central transverse section in the same blastoderm. The labelled cells are concentrated in a median section of the hypoblast (viewed with a fluorescence microscope). ep, epiblast; hyp, hypoblast. (D) A similar stage X blastoderm incubated to stage 3 H&H. (E) A transverse section of D. The central, labelled area in the lower layer is laterally expanded. In the non-labelled epiblast only the forming PS is totally labelled. A, B and D were photographed with reflected light on a dark background. C and E were photographed with fluorescent microscope.

A4 *(eight blastoderms)*

Eight blastoderms were operated upon at stage X and fixed at about stage 4 H&H (Fig. 4A). In all of them, the PS developed in front of the labelled implant. In the sections, all blastoderms were seen to have three germ layers. The intermediate layer, the mesoblast, has thus developed between stages 3 and 4 H&H (Fig. 2, column 4A).

The pattern of the labelled area in the lower layer was similar to that described for earlier stages, only that here the labelled area was still more extensive and had spread almost to the anterior and lateral margins of the lower layer. In the epiblast, the pattern was variable and included cases in which both the posterior and anterior ends of the PS were labelled, but separated by a non-labelled section. In others only the anterior end of the PS was labelled (Fig. 4C-E) while the rest was not (Fig. 4F-H), and in still others the PS was not labelled at all. The labelling pattern in the mesoblast fitted the epiblastic pattern very nicely and was complementary to it.

In those blastoderms in which both extremes of the PS were still labelled, the labelled mesoblastic cells were found near the midline relatively close to the PS. In the blastoderms in which only the anterior part of the PS (including Hensen's node) was labelled (Fig. 4C), labelled mesoblastic cells were found underneath the node (Fig. 4C) and anterior to it (Fig. 4B). In more posterior regions, the labelled mesoblastic cells were found to occupy a lateral position

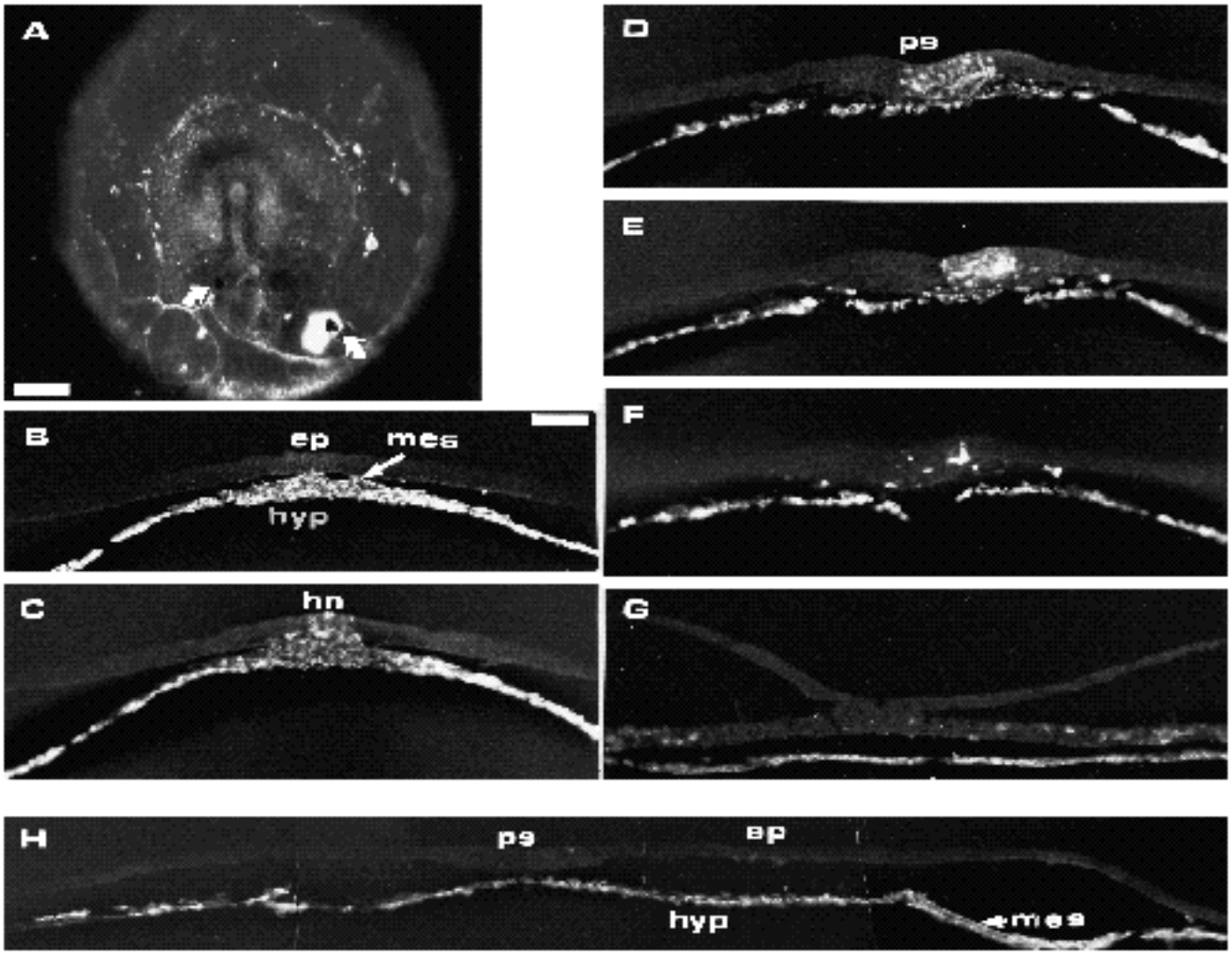

Fig. 4. (A) A blastoderm labelled at stage X and incubated to stage 4 H&H. The position of the carbon and carmine marks are indicated by curved arrows. Scale-bar - 1 mm. (B-G) Transverse sections of the above blastoderm from anterior to Hensen's node (B) to the posterior end of the PS (G). There is an extensive labelling of the hypoblast (hyp). The PS was labelled unilaterally (note the asymmetric positions of the carbon and carmine marks in A). The labelled cells of the PS invaginated faster in the posterior and central regions of the PS, and are therefore found in the lateral wings of the mesoblast (mes) (see G and H). Their place in the PS was already taken by non-labelled epiblastic cells (ep). In the anterior part of the PS, the unilateral label is quite obvious and the labelled gastrulating cells are anterior to the PS (B), under it (C) and spread somewhat laterally (C-E) (H) A posterior panoramic transverse section from a location between F and G. The hypoblast (hyp) is labelled, while the epiblast (ep) and PS are not. In the mesoblast (mes) labelled cells are found at very lateral positions while more median mesoblastic cells are not labelled and can hardly be seen A - photographed with reflected light on a dark background. B-H - photographed with fluorescence microscope. Scale bar on B - 250 μm. All the other sections are at the same magnification.

on both sides of the blastoderm while the mesoblastic cells underlying the PS were not labelled (Fig. 4G,H).

A5 *(seven blastoderms)*

In seven stage X and X+ blastoderms, the posterior section of MZ to be labelled and implanted was cut posterior to KS but very close to it. All blastoderms were incubated to stage 4 H&H and then fixed and sectioned. In all of them, the label was found in the originally labelled section and in some cases also the posterior part of the hypoblast contained labelled cells (Fig. 5E). The PS was not labelled but the mesoblast sometimes contained a few labelled cells (Fig. 2, column 5A).

Group B - *Experiments at stage XI E.G&K (Fig. 6)*

B1 *(one blastoderm)*

A posterior MZ fragment was cut out anterior to KS but close to it, from a stage XI blastoderm, labelled and put back into place. Development was stopped at stage XIII (Fig. 6, column 1B). In the serial sections, the label was found only in the implant.

B2 *(one blastoderm)*

This experiment was as in B1 but the blastoderm was fixed at stage XIV. The label was found mainly in the implant but, in addition, labelled cells were found to occupy a

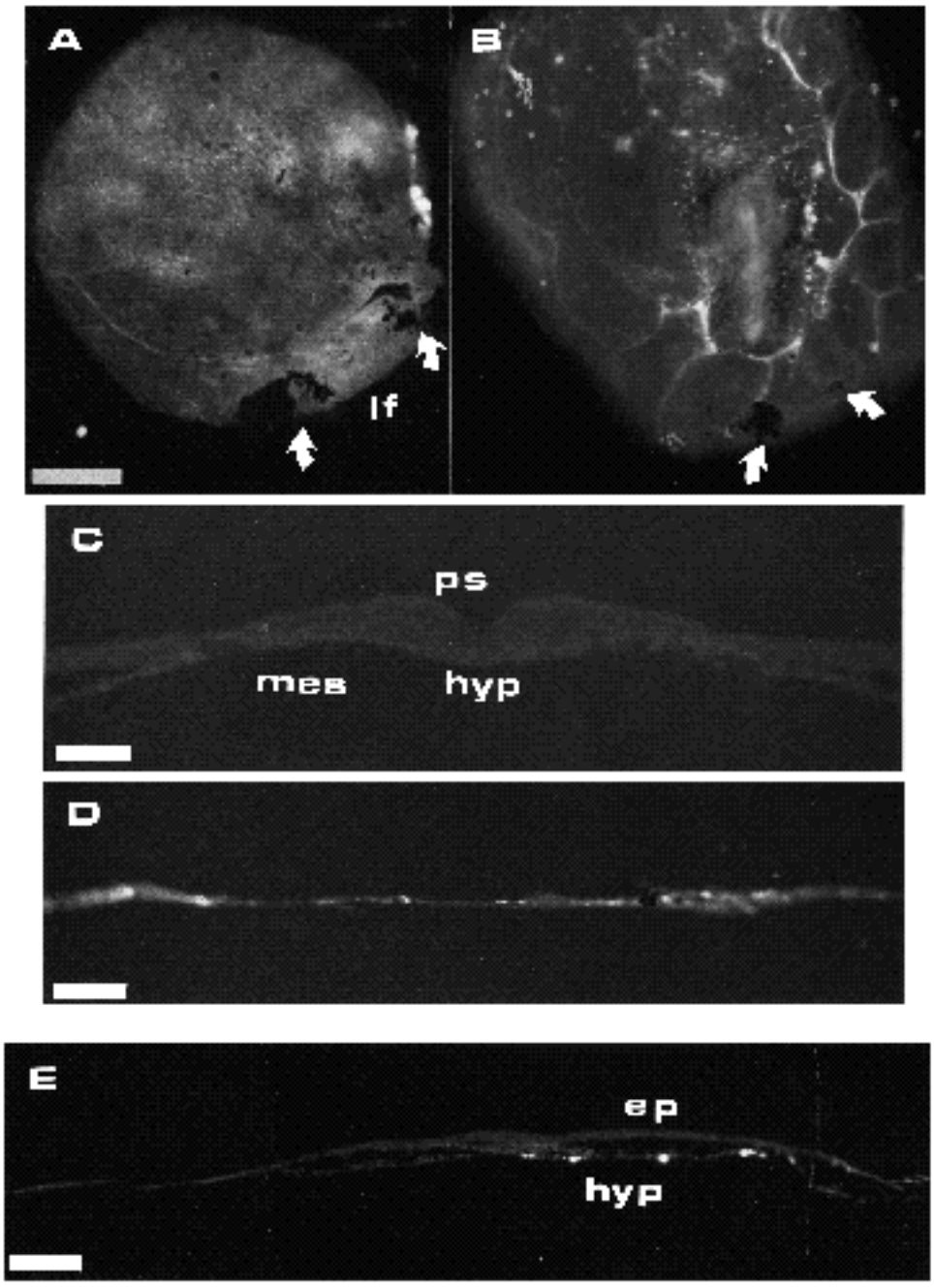

Fig. 5. (A) A stage X blastoderm. The labelled fragment (lf) is lateral to the midline (large carbon clump). (B) Same blastoderm after incubation to stage 4 H&H. (Note the posterior carbon label.) A and B are at the same magnification. Scale-bar on A - 1 mm. (C) The hypoblast (hyp), PS and mesoblast (mes) are not labelled. Scale bar - 250 μm. (D) Label was found only in a posterio-lateral section of the same blastoderm. Scale bar - 500 μm. (E) Transverse section in the posterior part of a stage 4 H&H blastoderm. The blastoderm was operated at stage X E.G&K and the labelled fragment did not include KS. The epiblast (ep), PS and mesoblast are not labelled. In the hypoblast (hyp), labelled cells are intermingled with non-labelled cells, probably definitive entodermal cells derived from the non-labelled PS. Scale bar - 500 μm. A and B photographed with reflected light on a dark background. C-E photographed with fluorescence microscope.

narrow median strip in the posterior third of the hypoblast. The epiblast, except for the implant, was not labelled (Fig. 6, column 2B).

B3 *(two blastoderms)*

The blastoderms were fixed at stage 3 H&H. Labelled cells were found in the posterior section of the lower layer. In one of the blastoderms, a small posterior section of the PS was also labelled (Fig. 6, column 3B).

B4 *(three blastoderms)*

The blastoderms were fixed at stage 4 H&H. In all three, a posterior section of the lower layer was found to be labelled. In one blastoderm, there were labelled cells in the posterior part of the PS as well as in the mesoblast while in the other two, neither the PS nor the mesoblast were labelled (Fig. 6, column 4B).

B5 *(8 blastoderms)*

The MZ fragment was cut posterior to and very close to KS. All 8 blastoderms were incubated to stage 4 H&H. In most of them, only the original implant was found labelled upon termination of the experiment (Fig. 6, column 5B). Only in three of the blastoderms were a few labelled cells found at the most posterior side of the lower layer and the mesoblast.

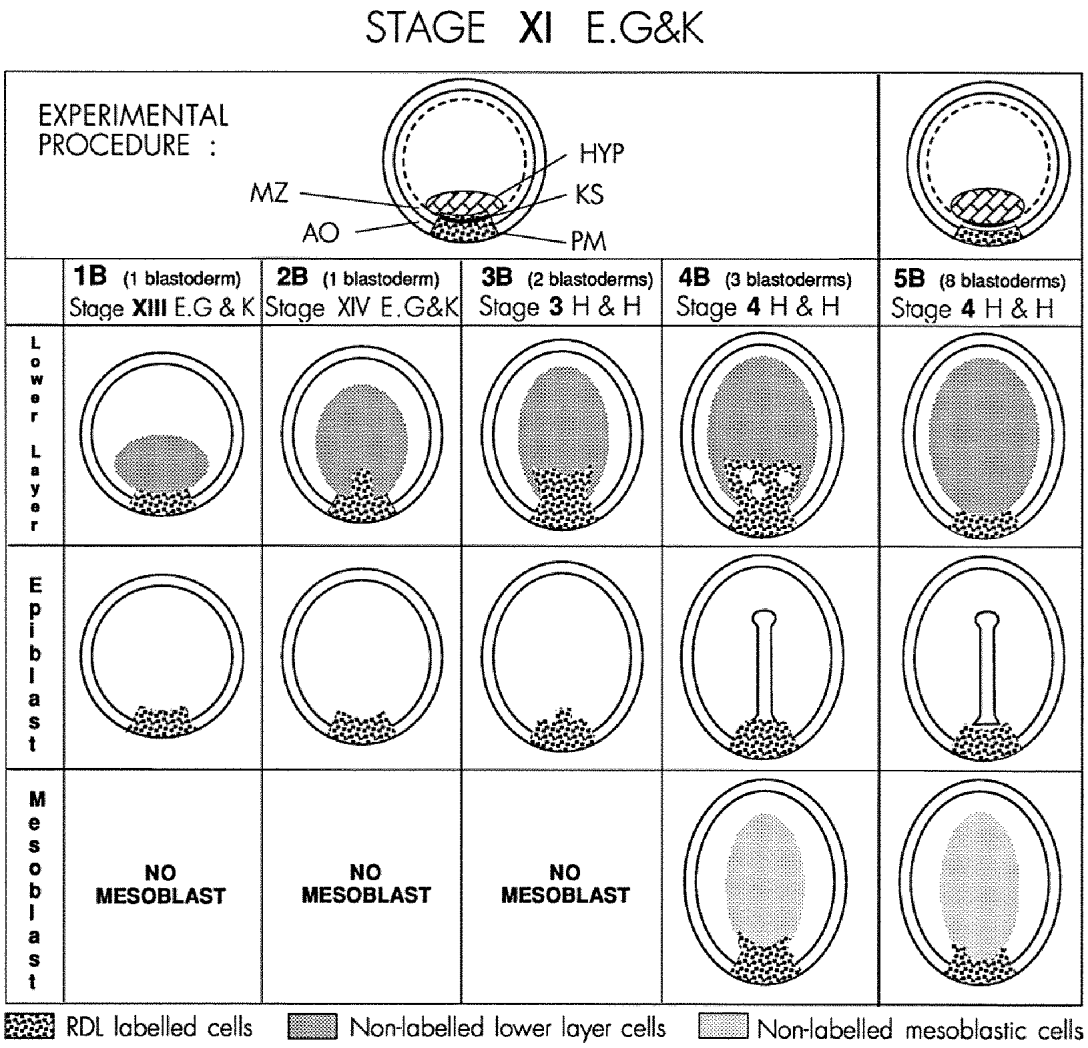

Fig. 6. Operations on stage XI blastoderms. The anterior border of the fragment was cut closer to KS than in stage X. AO, area opaca; HYP, hypoblast; MZ, marginal zone; PM, posterior marginal zone.

Group C - *Experiments at stage XII E.G&K*

In four stage XII blastoderms, a posterior section of the MZ including KS was cut out, labelled and returned to its original location. Incubation was terminated when the blastoderms reached stage 4 H&H. Some label was found in the posterior half of the lower layer. The PSs were never labelled, but occasionally some mesoblastic cells were found to be labelled.

No experiments were done with MZ fragments without KS since already in earlier stages the penetration of MZ cells into the germ layers seemed to have stopped.

Controls

Eighteen blastoderms of stages X (Fig. 2, right column) and XI were operated upon. A non-posterior marginal zone section (at 45°, 90° or 180° to the posterior point), similar in size to the posterior ones of the experimental series, was cut out, labelled, and then put back into the original blastoderm (Fig. 1E), which was further incubated to stage 4-5 H&H. The implants healed properly in nine blastoderms, in all of which the PS developed in front of the originally marked posterior side and not of the labelled implant. The serial sections revealed that the labelled cells stayed in the teritory of the implant (as judged by the position of the carbon and carmine marks, indicating its borders), and did not penetrate into any of the germ layers outside it (Fig. 5A-D).

In all the experimental series, the distribution of labelled cells was similar in blastoderms with and without the GWM fragment.

Discussion

The main difficulty in ascertaining the fate of the blastodermic cells, from stage X and on, was the lack of a reliable labelling method that would enable us to follow cell movements within the blastoderm. In the present study, we decided to label all the cells of defined marginal zone sections, with the fluorescent dye rhodamine-dextran-lysine (RDL), which was found to enter the cells and to become bound to the cell contents very quickly, and seems not to

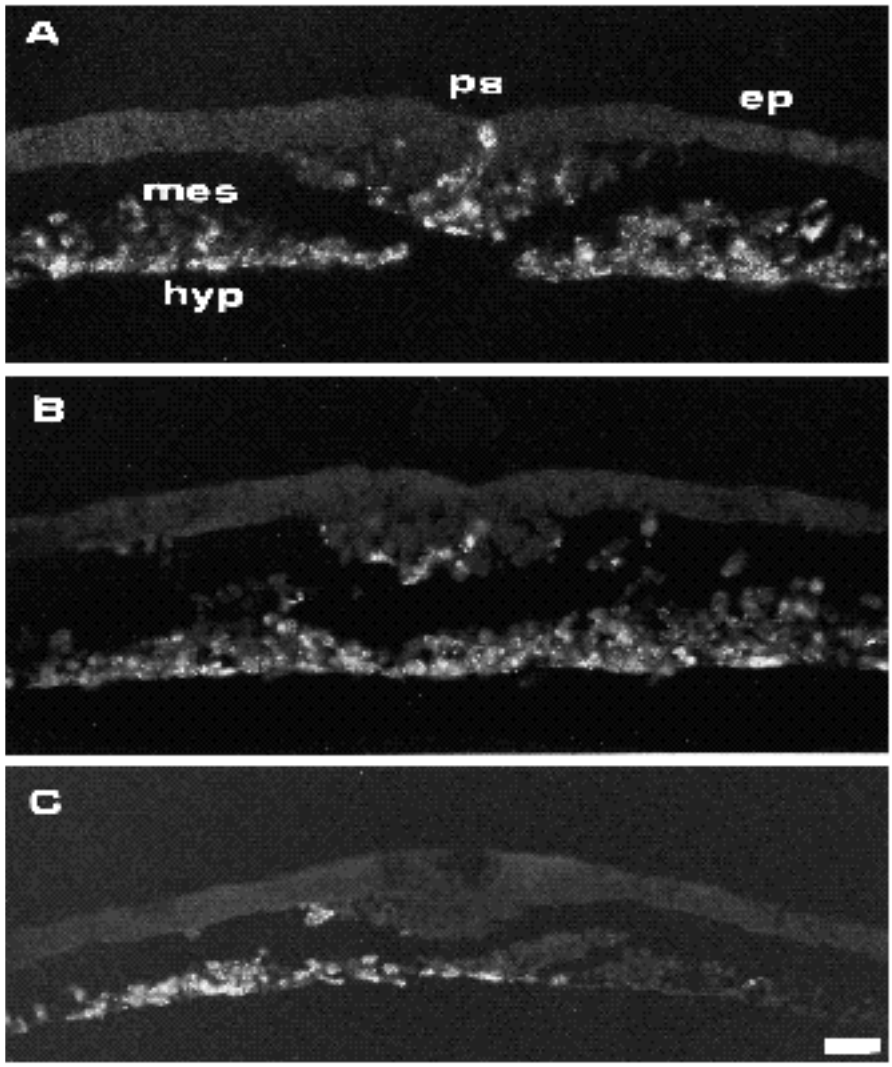

Fig. 7. (A-C; anterior to posterior). Higher magnification of three transverse sections in the central section of the PS of a stage 4 H&H blastoderm. The marginal zone + KS + and epiblastic strip (ep) were labelled at stage X E.G&K. Note distribution of labelled cells in the hypoblast (hyp), mesoblast (mes) and PS. Sections photographed with fluorescence microscope. White scale bar - 100 μm.

leak out. As a result, only the orignally labelled cells and their progeny were later found to contain the label. From the serial sections, it became clear that in the early stages (XII E.G&K to 3 H&H) the boundaries of labelled areas remained strictly defined, even after they shifted into new positions and changed their global shape. The sharp boundaries between labelled and non-labelled areas clearly persisted at the above stages (Fig. 3C,D). At later stages, however, a major change occurred when cells from stage 3 H&H and on started to migrate individually from the PS into the mesoblast. There, one could see that among neighbouring PS or mesoblastic cells, some were very brightly labelled while the rest were not (Fig. 7). Two conclusions can be drawn from this: (1) that the label does not diffuse from one cell to the other and (2) that the initial intensity of the label was so high that dilution was not very significant.

A second, very important aspect of our method was that the labelled sections were part of the host. This ensured that the graft was not only at the same developmental stage as the host, but that it was inserted into the exact place from which it was taken out. In such a case, even if there were a mistake in the correct identification of the posterior side (which can easily happen even to experienced observers), the error would be recognized as soon as the growth of either the hypoblast or the PS started. Such a blastoderm could then be either excluded or used as a laterally labelled control.

The third point that facilitated the interpretation of the results is that, before taking out the posterior section to be labelled, the marginal zone (senso stricto) was ventrally exposed by lifting up the flap of germ wall margin (a ventral, centrally directed extension of the germ wall) which was then excised and removed. Only in a few cases was the GWM left intact in order to see whether its presence would affect the distribution pattern of labelled cells.

Hypoblast formation

The overall picture based on all our experimental series' as the follows: During stages X-XII E.G&K, there is a massive penetration of a narrow front of marginal zone cells from Koller's sickle and into the forming hypoblast (see Figs 2 and 6). Most of the cells that are about to move into the hypoblast are already concentrated by stage X in Koller's sickle (compare columns 4A and 5A in Fig. 2). The future hypoblastic cells, which are not located in the

sickle at stage X, are still in the marginal zone posterior to it. They will enter the sickle by stage XI and will proceed (Stage XIV, Fig. 6, column 2B) to form the posterior section of the hypoblast (Fig. 6, column 4B). At stage XI, the marginal zone behind the sickle does not normally contribute cells any more to the hypoblast. Cells from lateral regions of the MZ do not normally participate in the formation of the hypoblast (Fig. 5C and Fig. 2 control). This does not exclude the possibility that in case of a deletion of the PM, which is the normal contributor to the hypoblast, more lateral sections of the MZ will take over and participate in hypoblast formation (Khaner and Eyal-Giladi, 1989).

The above conclusions also fit very nicely the experimental results of Khaner and Eyal-Giladi (1986, 1989) and Eyal-Giladi and Khaner (1989) who found that the PM with KS included, when transferred to a lateral position, will promote, in a stage X blastoderm, the formation of a lateral PS, while no PS will form at the original posterior side into which the removed lateral section of the MZ was inserted. However, when the same experiment was done on a stage XI blastoderm, two PSs developed, one lateral and one posterior. When a similar manipulation was done on a stage XII blastoderm, the laterally inserted PM did not cause PS formation and a normal PS was formed at the posterior location. Our interpretation was that at stage X the marginal zone cells, which are potential inducers of the PS, were still confined to their original position and therefore were transferred jointly to the lateral site from which they started to migrate into the hypoblast and induce a lateral PS. By stage XI, we thought that a substantial part of those cells had already moved into the initial hypoblast so that the population of potent PS-inducing cells would roughly be equally divided between the initial hypoblast which was left at the posterior side and the laterally transplanted PM. According to the above experimental results most of the potent inductive cells of PM origin should have left the marginal zone into the hypoblast by stage XII. The labelling experiments as summarized in Figs 2 and 6 show exactly the same pattern as predicted in the above mentioned papers. Indeed a very typical pattern of penetration of the PM cells into the hypoblast has been found. At the beginning (stages XII-XIII) they enter as a finger-like narrow projection pointing anteriorly, which pushes the alreay existing lower layer cells, of polyinvagination origin, to the sides (Figs 3C and 2, column 1A). From stage XIII E.G&K and on, the lower layer composed of cells from PM origin expands in both anterior and lateral directions (Figs 3E, 4B-H). It is not clear yet how this expansion is generated. We have probably to distinguish between the expansion until stage 3 H&H, and after that stage. Our labelling experiments have demonstrated that cell entry from the PM stops almost completely by stage XII, so that we have to consider other possible mechanisms that might contribute to the expansion. (1) Flattening of the finger-like cellular PM contribution in a lateral direction. In Fig. 3C, one can distinguish labelled cells in a more than one-cell-thick arrangement (compare to Fig. 3E). These cells might undergo a rearrangement into a single spread out layer. (2) A growth pressure from a posteriorly located mass of rapidly dividing cells. However, although cell division might play a role in normal development (Spratt, 1963), it was shown not to be absolutely essential for hypoblast formation. Weinberger and Brick (1982a,b) have inhibited division by treating blastoderms with colchicine, after which almost normal hypoblasts were formed.

Mitrani (1984) showed that a normal inductive hypoblastic layer formed when stage X blastoderms were submitted to X-ray irradiation with a dose of 6000 rads, which was believed to block cell division totally. We therefore have to assume that the hypoblast can form from the cells already present in a stage X blastoderm, even without further cell division. This probably refers to both cell populations that participate in hypoblast formation, the cells of PM origin as well as the ones that polyinvaginate.

After stage 3 H&H, there is probably an additional contribution of cells into the lower layer - definitive entodermal cells of PS origin. In those experiments in which the stage X labelled PM section also included a part of the CD anterior to KS, the PS of stage 3 H&H was found to be entirely labelled. Therefore at the onset of gastrulation, which started shortly afterwards, the labelled invaginating cells penetrated both the mesoblast and also the lower layer. Cells of PS origin have already been found to enter randomly into the lower layer and form a mosaic of hypoblastic and entodermal cells (Azar and Eyal-Giladi 1983). The penetrating cells from PS origin, which will later sort out to form the definitive entoderm (Sanders et al., 1978), will therefore also contribute to the lower layer's expansion. Those cells cannot be distinguished in most of our experiments from the original hypoblastic cells. However, their presence can be proven if we compare an experiment from series 4A with an experiment from series 5A (Fig. 2). When a 4A experimental blastoderm reached stage 4 H&H almost all the cells in the centeral part of the lower layer (hypoblastic plus definitive endoblast) were labelled (see Fig. 4, all transverse sections). In contrast, in 5A experiments in which the labelled part did not include KS and the epiblastic part anterior to it (which will form the PS), only the posterior part of the lower layer contained labelled cells intermingled with non-labelled ones, which are probably definitive entodermal cells from a non-labelled PS origin (Fig. 3E and Fig. 2, column 5A, the white spots in the lower layer).

The formation of the hypoblast as we see it is quite different from the picture drawn by Stern (1990) and Stern and Canning (1990). We believe that the results and conclusions as presented in the above mentioned papers can be explained by the operation technique and the manner in which the blastoderms have been separated from the underlying yolk (personal communication). We think that in these experiments the PM as well as KS were not exposed and the early developmental stages (X-XI) could not have been accurately determined. What was probably labelled in the above experiments were as follows. (1) The anterior part of a stage XI hypoblast, which has advanced anteriorly beyond the cover of the anterior border of the GWM and the yolk adhering to it. (2) The epiblastic strip anterior to KS, which also has not been exposed. In the meantime, a better understanding of the blastodermic anatomy has been reached and agreed upon with Stern and other investigators in the field (Bellairs et al. personal communication) which

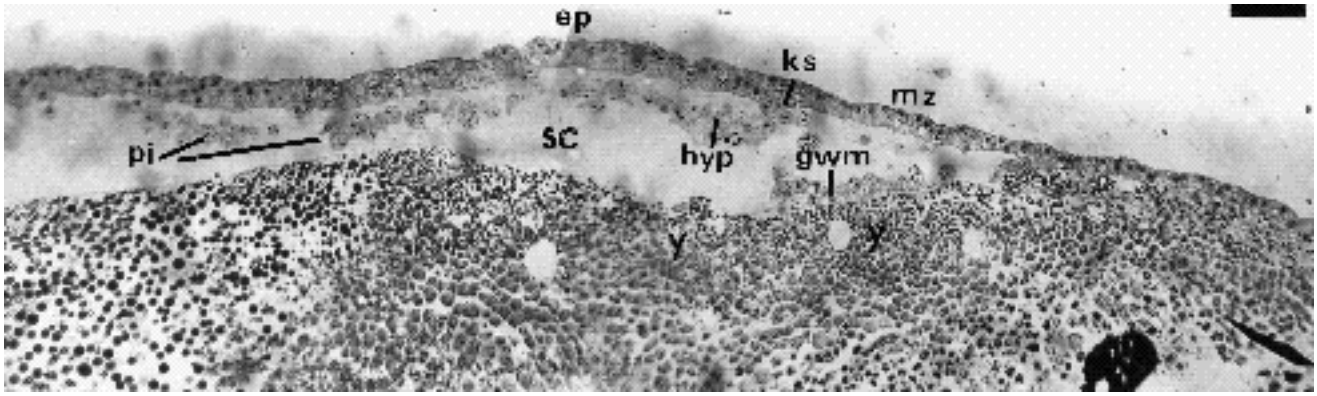

Fig. 8. Sagittal section of a stage XI E.G&K blastoderm, fixed without opening the subgerminal cavity to preserve normal topographic relations between the different parts. The marginal zone (mz), germ wall margin (gwm), Koller's sickle (ks), the posterior hypoblastic portion continuous to KS (hyp), as well as the more anteriorly situated polyinvagination islands (pi) are clearly seen. The carbon spot was put at the predicted posterior side prior to fixation. sc, sub-blastodermic cavity; y, yolk. Scale bar - 100 μm.

will avoid misunderstandings in the future. Meanwhile, in order to clarify the topographical relationships between the different components in this important region, we have enclosed a microscopic sagittal section of the posterior region (Fig. 8).

Our experiments were done on two parallel series, one in which the explants were cut anterior to Koller's sickle and the other in which they were cut posterior to it. This made it possible to show that the posterior marginal zone *does not* contribute to the PS, but does contribute to the hypoblast. According to our results the penetration pattern of labelled cells into the hypoblast was not changed in those cases in which the GWM was included in the fragment. However, in order to determine whether the GWM does also contribute cells to the hypoblast, as claimed by Stern (1990), some additional experiments should be done.

However, we have found that the entire cell population which forms the stage 3 H&H PS (Fig. 3D-E), derives from a very narrow epiblastic strip anterior to KS of a stage X blastoderm. The cells that form the PS therefore do not collect from cells randomly distributed in the epiblast as claimed by Stern and Canning (1990) (see also Eyal-Giladi, 1991). We believe that these epiblastic cells after being induced very early by the advancing posterior marginal zone cells, quickly rearrange and by stage 2 H&H shoot anteriorly to form a median thickened primordium of the PS. However, most of those early PS cells invaginate into the mesoblast and endoblast already by stage 4 H&H and are replaced by non-labeled epiblastic cells situated around the initial PS. The cells newly recruited into the PS are probably induced somewhat later in development than the initial ones, by the progressing cells of PM origin. Our results support the old concensus among embryologists that the PS of avians is homologous to the blastopore of amphibians and that the avian mesoblast gastrulates via the PS.

Another interesting aspect is demonstrated by the blastoderm of Fig. 4. In this blastoderm, the PS has been labelled only unilaterally; however, labelled mesoblastic cells are found on both sides of the blastoderm although there are more of them on the side where the PS is labelled. This means that the gastrulating cells do not migrate only on the side of the PS from which they came, but can also migrate to the contra lateral side. Similar observations were also made by Rosenquist (1966). Fig. 4, among others, can form a good starting point for the formulation of the developmental model into which all the known data can be integrated. There seems to be a behavioral as well as a shape difference between the anlage of cells of the epiblastic strip which will move into the PS and the anlage of PM cells moving into the lower layer. As can be seen from Fig. 3A the width (left to right) of both the epiblastic section anterior to KS and of the PM, when labelled during the experiment, is almost equal (the PM is slightly wider). However, the PM section, which is situated more posteriorly, starts to move first (stage XI) in a narrow front (Fig. 9A) and to

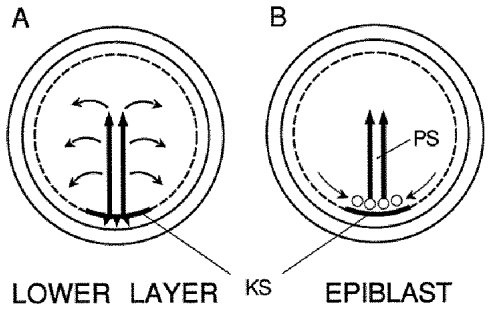

Fig. 9. A model representing movements of the cellular anlagae within the upper and lower germ layers. (A) Movements of the inductive cells of PM origin (black triangles) into the median line of the forming hypoblast starting at stage X E.G&K. The movement forwards (thick arrows) continues until stage XII-XIII and is then changed into a lateral expansion of the anlage (thin arrows). (B) Movement of the PS anlage (circles) initially situated anterior to Koller's sickle. The movement starts at stage XII-XIII at the centromedian point of the anlage (thick arrows) after being induced by the earlier migrating PM cells underneath. The median position of initial centromedian section is gradually occupied after its anterior migration by more laterally situated epiblastic cells (thin arrows). The last cells to move into the central area and be induced there will form the most posterior section of the PS.

advance into the forming hypoblast under the central portion of the epiblastic strip, which is at that time still stationary. The moving front continues to advance in the same pattern until stage XII-XIII (compare hypoblast and epiblast in Fig. 2, 2A). Around stage XIII, the cells of PM origin start to spread also laterally and occupy an extensive part of the lower layer. A fountain-like movement of cells in the lower layer was already mentioned by Spratt and Haas (1960).

A rather limited central section of the PM seemed to supply the labelled cells to the hypoblast. However, for the epiblastic strip the picture is different. In many cases the PS was found to be labelled unilaterally for its entire length when the lower layer underneath was normally labelled. The epiblastic strip (Fig. 9B) contributing cells to the PS must therefore be quite narrow in an anterio-posterior direction and wider laterally than the PM section contributing to the hypoblast. This assumption must however still be verified experimentally by careful labelling of the epiblastic strip anterior to KS.

Concerning the temporal relationship between the migrating PM and future PS cells we suggest the following scenario. When the cells of PM origin leave KS and move anteriorly into the narrow median section of the forming hypoblast, they interact with the central portion of the epiblastic strip (PS anlage) and induce it to form the anterior tip of the PS which is Hensen's node. The induction is translated after a certain lag period into the anterior migration of this central area while its place is subsequently being occupied by the more laterally situated cells of the strip. Those lateral cells converge centrally into a position that would allow them to be induced by the underlying hypoblastic cells of PM origin to form more posterior sections of the PS.

The patterns of cell movements within each of the two germ layers concerned are therefore very different (Fig. 9). While in the hypoblast the cells penetrate like an arrowhead and later on spread out like a fountain, in the epiblast the central cells in the strip anterior to KS move anteriorly to form the narrow elongated PS, while the more laterally situated cells in the posterior epiblastic strip converge from the sides to the center and after reaching the midline continue to move anteriorly. Only after the blastoderm has reached stage 3+ H&H and the cells of its PS start to gastrulate, do additional, even later induced epiblastic cells gradually populate the PS.

Many thanks are due to Mr Peter Yanai for synthesizing the RDL, to Dr O. Khaner who started to develop the labelling method and to both Drs O. Khaner and M. Ginsburg for very useful discussions and remarks. We are also very much obliged to Dr C. Stern for his interest and for the good discussions that we had.

This study was supported by research grant N. 85-00230 received from the United States-Israel Binational Science Foundation.

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.

Azar, Y. and Eyal-Giladi, H. (1983). The retention of primary hypoblastic cells underneath the developing primitive streak allows for their prolonged inductive influence. *J. Embryol. Exp. Morph.* **77**, 143-151.

Bellairs, R. et al. Concensus terminology for avian embryos during the early stages of development (personal communication).

Duval, M. (1884). De la formation du blastoderme dans l'oeuf d'oiseau. *Ann. Sci. Nat. VIe serie, Zool.* **18**, 1-208.

Duval, M. (1888). Atlas d'Embryologie. Paris.

Eyal-Giladi, H. (1984). The gradual establishment of cell commitments during the early stages of chick development. *Cell Differentiation* **14**, 245-255.

Eyal-Giladi, H. (1991). The early embryonic development of the chick, as an epigenetic process. *Crit. Rev. Poultry Biol.* **3**, 143-166.

Eyal-Giladi, H. (1992). The avian marginal zone and its role in early development. In *Formation and Differentiation of Early Embryonic Mesoderm*, 9-21.

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

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

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

His, W. (1868). *Untersuchungen uber die erste Anlage des Wirbeltierleibes.* Leipzig.

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. Coordinative effect of induction and inhibition. *Dev. Biol.* **134**, 206-214.

Kochav, S., Ginsburg, M. and Eyal-Giladi, H. (1980). From cleavage to primitive streak formation: A complementary normal table and a new look at the first stages of the development of the chick. II. Microscopic anatomy and cell population dynamics. *Dev. Biol.* **79**, 296-308.

Krotoski, D. M., Fraser, S. E. and Bonner-Fraser, M. (1988). Mapping of neural crest pathways in *Xenopus laevis* using inter- and intra-specific cell markers. *Dev. Biol.* **127**, 119-132.

Merbach, H. (1936). Beobachtungen an der Keimscheibe des Huhnchens vor dem Erscheinen des Primitivstreifens. *Z. ges. Anat. I. Z. Anat. Entw. Gesch.* **104**, 635-652.

Mitrani, E. (1984). Mitosis in the formation and function of the primary hypoblast of the chick. *Roux' Arch. Dev. Biol.* **193**, 402-405.

Nowack, K. (1902). Neue Untersuchungen uber die Bildung der beiden primaren Keimblatter und die Entstehung des Primitivstreifens beim Huhnerembryo. Inaugural-Dissertation (E. Ebering, Berlin).

Pasteels, J. (1945). On the formation of the primary entoderm of the duck (Anas domestica) and on the significance of hte bilaminar embryo in birds. *Anat. Rec.* **93**, 5-21.

Patterson, J. T. (1909) Gastrulation in the pigeon's egg. A morphological and experimental study. *J. Morph.* **20**, 65-123.

Rosenquist, G. C. (1966). A radioautographic study of labelled grafts in the chick blastoderm. Development from primitive- streak stages to stage 12. *Contrib. Embryol. Carnegie Inst.* **38**, 71-110.

Sanders, E. J., Bellairs, R. and Portch, P. A. (1978). In vivo and in vitro studies on the hypoblast and definitive endoblast of avian embryos. *J. Embryol. Exp. Morph.* **46**, 187-205.

Spratt, N. T. (1963). Role of the substratum, supracellular continuity and differential growth in morphogenetic cell movements. *Dev. Biol.* **7**, 51-63.

Spratt, N. T. and Haas, H. (1960). Morphogenetic movements on the lower surface of the unincubated and early chick blastoderm. *J. Exp. Zool.* **144**, 139-157.

Spratt, N. T. and Haas, H. (1965). Germ layer formation and the role of the primitive streak in the chick. I. Basic architecture and morphogenetic tissue movements. *J. Exp. Zool.* **158**, 9-38.

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.

Stern, C. D. and Canning, D. R. (1990). Origin of cells giving rise to mesoderm and endoderm in chick embryo. *Nature* **343**, 273-275.

Vakaet, L. (1962). Some new data concerning the formation of the definitive endoblast in the chick embryo. *J. Embryol. Exp. Morph.* **10**, 38-56.

Weinberger, C. and Brick, I. (1982a). Primary hypoblast development in the chick. I. Scanning Electron Microscopy of normal development. *Roux' Arch. Dev. Biol.* **191**, 119-126.

Weinberger, C. and Brick, I. (1982b). Primary hypoblast development in the chick. II. The role of cell division. *Roux' Arch. Dev. Biol.* **191**, 127-133.

(Accepted 10 July 1992)