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Replacement of posterior by anterior endoderm reduces sterility in embryos from inverted eggs of *Xenopus laevis*

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SUMMARY

The genital ridges of *Xenopus laevis* tadpoles reared from eggs kept in an inverted position contain less than 40 % of the number of primordial germ cells (PGCs) of controls (Cleine & Dixon, 1985). It has been suggested that this reduction is caused by the germ cells' ectopic position in the anterior endoderm of larvae from inverted eggs, from where they may be unable to migrate into the genital ridges (Cleine & Dixon, 1985). This hypothesis is tested here by interchanging anterior and posterior endodermal grafts between pairs of inverted embryos at the early tailbud stage. Replacement of anterior by posterior endoderm has no effect but replacement of posterior by anterior endoderm increases the number of PGCs in the genital ridges and significantly reduces the proportion of sterile embryos. In a control series, in which the same type of grafting was done with normal embryos, replacement of posterior by anterior endoderm reduced the number of germ cells to almost zero, but replacement of anterior by posterior endoderm nearly doubled it. These findings are explained in terms of the distribution of the germ cells in the endoderm at the time of grafting. The results firstly show that the position of the germ cells is crucial to successful migration and secondly they support the notion that germ plasm has a determinative role during early germ cell differentiation.

INTRODUCTION

Eggs of *X. laevis*, kept in an off-axis orientation, develop into embryos with significantly reduced numbers of primordial germ cells in their genital ridges (Neff, Malacinski, Wakahara & Jurand, 1983; Thomas *et al.* 1983; Wakahara, Neff & Malacinski, 1984; Cleine & Dixon, 1985).

In a previous study (Cleine & Dixon, 1985) it was shown that embryos of *X. laevis*, reared from eggs that were kept in an upside-down (inverted) orientation, had less than 40 % of the number of primordial germ cells in controls. Reconstruction from serial sections showed that in such eggs the heavy yolk slips along one side largely into the pigmented hemisphere except for a 20–30 μm thick layer that adheres to the cortex. It was further shown that germ plasm, a

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cytoplasmic inclusion specific for the early germ cell lineage of anurans (Bounoure, 1934; Whitington & Dixon, 1975), stays in its original subcortical position at the unpigmented pole of the inverted embryo during the cleavage stages (Cleine & Dixon, 1985). The cleavage pattern, which adjusts itself to the new yolk distribution, establishes a new animal–vegetal polarity which no longer corresponds to the original pigment pattern of dark and light hemispheres. The new cleavage pattern means that blastomeres in the dorsal equatorial region inherit the germ plasm in contrast to normal embryos where it is found in blastomeres around the vegetal pole. Reconstruction further showed that in inverted embryos the germ-plasm-containing cells localize around the dorsal lip of the blastopore and during gastrulation invaginate further than in normal embryos (Cleine & Dixon, 1985). In the tailbud larva most of the germ-plasm-containing cells (70–80 %) are found throughout the anterior half of the endoderm (Cleine & Dixon, 1985) unlike in controls where they form a few clusters in the posterior endoderm (Blackler & Fischberg, 1961; Subtelny & Penkala, 1984; Cleine & Dixon, 1985).

It was speculated that the abnormal distribution of the cells with germ plasm is the cause of the reduced number of primordial germ cells in inverted embryos (Cleine & Dixon, 1985). Normally the primordial germ cells migrate out of the endoderm to the prospective genital ridges on both sides of the dorsal root of the mesentery in the posterior part of the coelom (reviews: Nieuwkoop & Sutasurya, 1979; Heasman & Wylie, 1983). Apparently most, if not all primordial germ cells take part in this migration: their number is fixed because they become mitotically inactive by stage 36 (Dziadek & Dixon, 1977) and in the preceding period (stage 25–35) their numbers in the endoderm are 70–90 % of those later found in the genital ridges (Cleine & Dixon, 1985; Cleine, unpublished results). For a detailed account of the number of cells in the germ cell lineage, see Dixon, 1981.

Primordial germ cells in the anterior endoderm of inverted embryos may be too far removed from the genital ridges to reach them. To test the hypothesis that the position of the primordial germ cells in the endoderm is crucial to successful migration two series of graftings were performed, one with inverted embryos and one with normal embryos, in which the anterior and posterior halves of the endoderm were interchanged.

The main results are that replacement of posterior by anterior endoderm in inverted embryos gives rise to an increase in the number of primordial germ cells in the genital ridges and to a significant decrease in the proportion of sterile embryos. (The term 'sterile' is applied to embryos without any primordial germ cells in their genital ridges. Unlike u.v.-irradiated embryos which often show a transient 'sterility' because the germ cells migrate into the genital ridges later than normal (Züst & Dixon, 1977; Williams & Smith, 1984), inverted embryos have no delayed migration (Cleine & Dixon, 1985).)

Rather unexpectedly, the number of primordial germ cells in normal embryos almost doubles if anterior endoderm is replaced by posterior endoderm. The results can be explained in terms of germ cell distribution in the endoderm at the

time of grafting and they provide supportive evidence for the determinative role of germ plasm during early germ cell differentiation.

MATERIALS AND METHODS

Rotation

Dejellied eggs of *X. laevis* were rotated in a 5–7 % Ficoll solution at 13°C as described by Cleine & Dixon (1985). Only rotations in two steps of 90° were carried out because the effect of this type of rotation on the number of primordial germ cells was most marked (Cleine & Dixon, 1985). Embryos obtained from eggs rotated in this way are referred to as 'inverted' throughout.

Grafting procedure

All manipulations were performed at room temperature.

Embryos at stage 23–24 (Nieuwkoop & Faber, 1967) were demembranated with watchmaker's forceps in full-strength MMR saline (see Cleine & Dixon, 1985) and kept therein until they were operated on at stage 25–28. Demembranating a few hours before operation gave the embryos time to straighten and to stretch, which facilitated the operation.

During the grafting procedure larvae were kept in sterilized MMR which contained 10^5 i.u. penicillin l^{-1} and 100 mg streptomycin l^{-1}. Larvae were immobilized prior to grafting by immersing them for 20–30 s in a dish with MMR to which a few crystals of MS 222 (tricaine methane sulphonate, Calbiochem) were added. The grafting operation was adapted from the method originally developed by Blackler & Fischberg (1961) and since used in a number of other studies (Blackler & Gecking, 1972; Subtelny & Penkala, 1984).

Larvae were operated on in 5 cm plastic Petri dishes on a layer of agarose (2 % in MMR, Calbiochem) in which V-shaped grooves (approximately 0·5 mm deep and 0·2 mm wide) had been excised. Parts of the endodermal region, including the surrounding mesoderm and epidermis, were excised with microscalpels made of chips of razor blade mounted on a glass rod with epoxy resin (Araldite). Larvae were then put in the grooves with their ventral sides upwards and slightly above the surface of the agarose layer. Grafts from the endodermal region of two embryos were exchanged. The grafts were held in position by a glass rod (about 1 cm long and 0·2 mm in diameter) laid upon the graft at right angles to the groove. The rods were removed about 15 min after the operation. Glass rods were found to be more satisfactory than wide glass bridges which tended to push host and graft tissue apart.

Histology and counting of primordial germ cells

Tadpoles at stage 46 were fixed in Smith's Fixative and embedded in Paraplast. Primordial germ cells were counted on 8 μm serial transverse sections stained with haematoxylin–aurantia–eosin as described by Cleine & Dixon (1985).

RESULTS

Types of grafting

Anterior and posterior endodermal regions were interchanged either between non-rotated embryos or between inverted ones, giving rise to two sets of reciprocal pairs: normal-aa and normal-pp; inverted-aa and inverted-pp (Fig. 1). No grafts were exchanged between normal and inverted embryos.

Healing of the grafts and development

Grafts healed quickly within 10 to 15 min after operation. The embryos were left in the operation dish in full-strength MMR for about 18 h. About stage-36 larvae with well-healed grafts were transferred to filtered tapwater. The criterion for well-healed grafts was that there was no gap between host and graft tissue (see Fig. 2). Thus there was one continuous endodermal mass in which PGCs could migrate freely.

Not all larvae that initially healed well, developed normally (Table 1). In a few cases substantial parts of the graft were lost, but more frequently the tadpoles showed an abnormal morphology with microcephaly, swelling of the body cavity

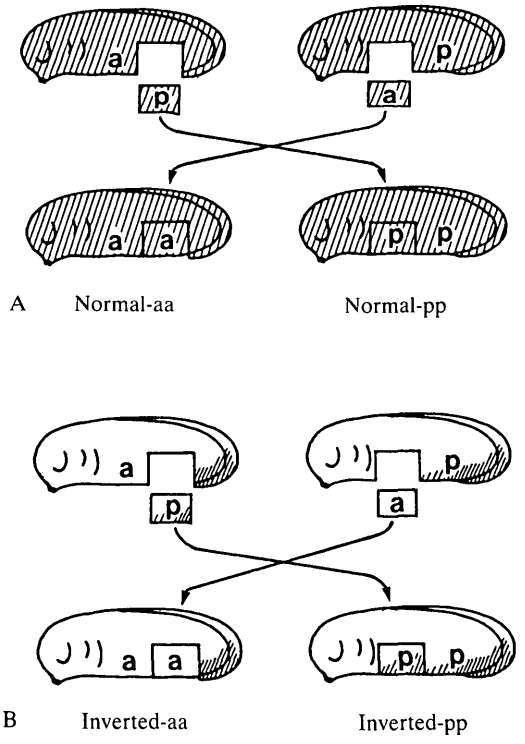

Fig. 1. Interchange of anterior and posterior endodermal grafts. (A) Normal non-rotated larvae. (B) Larvae from inverted eggs.

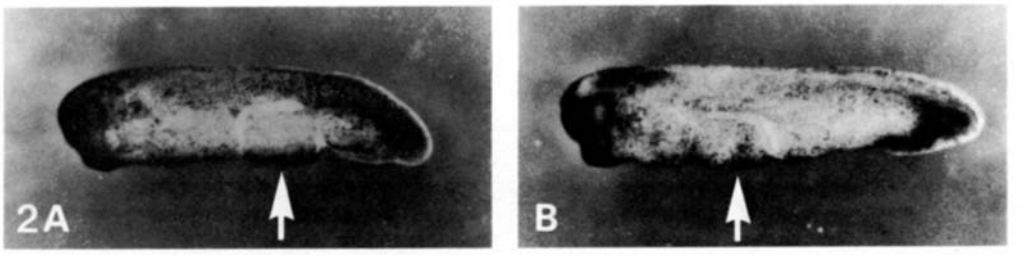

Fig. 2. Pair of larvae at stage 29/30 about 4 h after the grafts (arrows) were interchanged at stage 26. (A) Normal-aa; (B) normal-pp. Length of larvae: approx. 4·5 mm.

Table 1. *Development of grafted embryos*

	No. of graftings	Good healing stage 36	Normal stage 46	Success rate (%)
Normal-aa	110	34	24	22
Normal-pp	110	36	31	28
Inverted-aa	66	35	14	21
Inverted-pp	66	35	13	20

and extrusion of yolky cell masses from the intestine. These larvae were discarded. Only larvae in which the grafts healed well and which subsequently exhibited a normal morphology were used. The proportions of embryos that developed successfully were in the range of 20–30 % (Table 1). Examples of 'abnormalities' that were not considered justification for exclusion were a slight difference in the coiling patterns of the gut, slight enlargement of the body cavity, some hypertrophy of the nephric duct or the formation of additional mesenteries. All the larvae from which data were taken appeared normal in the region of the genital ridges, with a well-developed single dorsal mesentery.

The number of primordial germ cells reaching the genital ridges

To test the effect of the operation on the number of primordial germ cells (PGCs) posterior endoderm was interchanged between normal larvae at stage 26 and the number of PGCs that had migrated into the genital ridges were scored at stage 46. In eleven such operated embryos the number of germ cells was $10{\cdot}5 \pm 7{\cdot}9$ compared to $11{\cdot}6 \pm 8{\cdot}0$ in controls. This difference is not significant (t-test, $P > 0{\cdot}2$) which shows that the operation itself caused no significant loss of germ cells.

The number of PGCs in the genital ridges of the experimental groups are given in Table 2. The average number in controls (11·6) was somewhat lower than previously found (Cleine & Dixon, 1985) and was largely due to an inherent sterility in this batch of embryos of which 20 % of the controls had no germ cells.

In inverted embryos sterility was greatly enhanced; 68 % of this group showed no germ cells. The larvae that were not sterile had on average a lower number of germ cells than those in controls (Fig. 3A,D).

Normal-aa larvae were mostly sterile (75 %) and the remaining embryos had very few germ cells (Fig. 3B). This indicates that the vast majority of the host's own germ cells were successfully removed and that the anterior endoderm of normal embryos contains few or no germ cells.

The reciprocally treated group, the normal-pp larvae, had almost double the number of germ cells of controls (Table 2). This indicates that in normal embryos the germ cells of the graft are able to reach the genital ridges despite its abnormal anterior position.

In inverted-aa larvae the average number of germ cells was higher and sterility significantly lower than in the inverted embryos (Fig. 3E). The conclusions to be drawn from this are twofold. Firstly it agrees with the finding that the anterior

endoderm of inverted embryos contains most of the germ cells (Cleine & Dixon, 1985) and secondly it shows that these anterior germ cells can only migrate to their proper destination after transplantation to the posterior region.

The reciprocally treated group, the inverted-pp larvae, showed a similar number of germ cells and sterility as the inverted ones (Table 2, Fig. 3D,F). This strengthens the suggestion that in inverted embryos only the posteriorly located germ cells migrate to the genital ridges (Cleine & Dixon, 1975).

Table 2. *Number of primordial germ cells in genital ridges and number of sterile embryos at tadpole stage 46*

	Number of PGCs (Mean ± S.D.)	Number of embryos	
		Total	Sterile (%)
Controls	11·6 ± 8·0	20	4 (20)
Inverted	2·1 ± 3·9	19	13 (68)
Normal-aa	0·5 ± 1·0	20	15 (75)
Normal-pp	21·2 ± 7·6	19	0 (0)
Inverted-aa	6·4 ± 6·3	14	2 (14)
Inverted-pp	2·2 ± 2·9	13	8 (62)

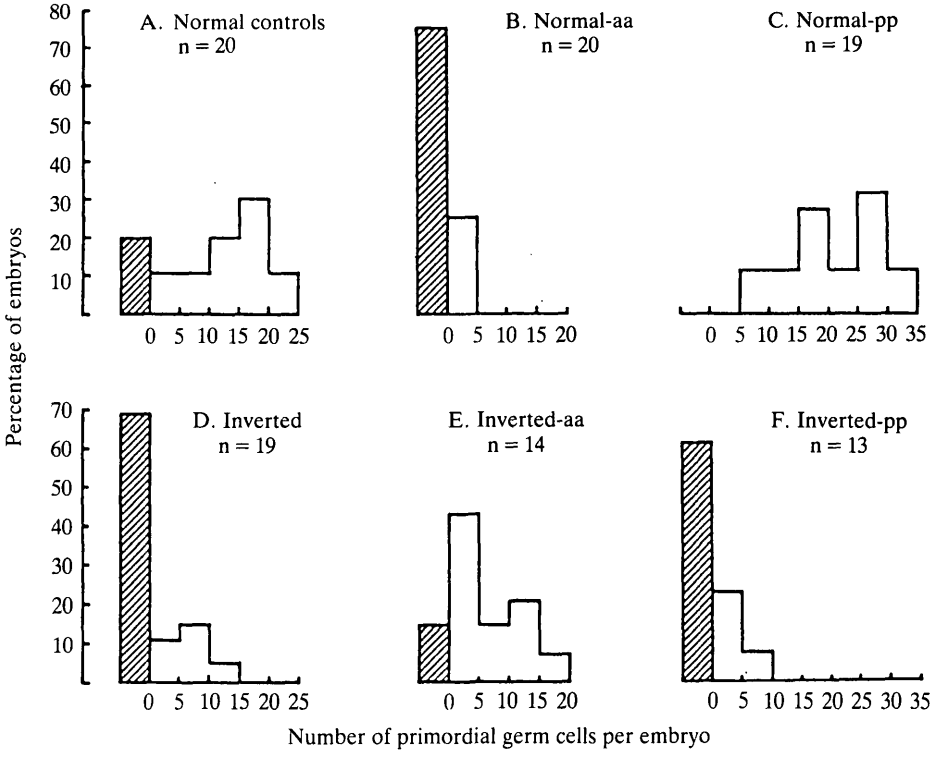

Fig. 3. Frequency distribution of the number of primordial germ cells (PGCs) in the genital ridges of stage-46 tadpoles. Horizontal axis, number of PGCs per embryo; vertical axis, percentage of embryos. Cross-hatched bars represent the proportion of sterile embryos. n, number of embryos.

The effect of grafting on sterility in inverted embryos

The high frequency of sterility in inverted embryos is responsible for the skew distribution of the number of germ cells (Fig. 3D,F). This, together with the relatively small numbers of embryos that are scored, means that differences in the mean cannot be directly assessed by conventional statistical methods such as the *t*-test or Wilcoxon's Rank Sum test.

Increase or decrease in the frequency of sterile embryos was the most conspicuous response to rotation and grafting, and therefore the proportion of sterile embryos has to be considered as a major variable in each experimental group. Differences between groups based on the numbers of sterile *versus* non-sterile embryos were evaluated by Fisher's Exact Test for 2×2 tables (Fisher, 1958) and are presented in Table 3. The *P*-values show that sterility is significantly higher in inverted than in control embryos. Replacement of anterior by posterior endoderm in inverted embryos (inverted-pp larvae) has no effect, but replacement of posterior by anterior endoderm (inverted-aa larvae) reduces the sterility to a level not significantly different from that of controls.

Conclusion

Taken together the results indicate that the main cause for the low number of PGCs and the high sterility in inverted embryos is the germ cells' ectopic position in the anterior endoderm from where they are unable to migrate to the posteriorly situated genital ridges.

DISCUSSION

Distribution and migration of primordial germ cells

Anterior and posterior endodermal grafts were interchanged between non-rotated or between inverted embryos to investigate the distribution of the primordial germ cells and their ability to migrate to the genital ridges.

The results of grafting with non-rotated embryos fully agree with the earlier reported finding that PGCs are normally localized in the posterior endoderm (Blackler & Fischberg, 1961; Subtelny & Penkala, 1984; Cleine & Dixon, 1985). The results with inverted embryos indicate that in these embryos most of the germ

Table 3. *Differences in sterility assessed by Fisher's Exact Test for 2×2 tables*

Groups compared	Probability *P*	Significance
Controls–inverted	0·003	S
Controls–inverted-pp	0·020	S
Controls–inverted-aa	0·518	NS
Inverted–inverted-aa	0·003	S
Inverted–inverted-pp	0·783	NS

S, significant; NS, not significant.

cells are localized in the anterior endoderm from where they cannot migrate to the genital ridges.

However, with respect to the germ cells' ability to migrate from an anterior position, there seems to be a discrepancy between inverted and normal-pp larvae. In the first group germ cells in an anterior position did not migrate to the genital ridges whereas in the second group they did. This result may reflect some intrinsic defect in inverted embryos with respect to the mechanisms that surround germ cell migration. But such a defect, if any, cannot be severe since the germ cells did migrate after transplantation to the posterior region. A more satisfactory explanation can be sought in the difference in anteroposterior distribution of the germ cells at the time of grafting. In inverted embryos most of the germ cells are found throughout the anterior half of the endoderm, widely separated from each other (Cleine & Dixon, 1985).

In normal embryos, however, germ cells form clusters in the posterior endoderm (Blackler & Fischberg, 1961; Subtelny & Penkala, 1984; Cleine & Dixon, 1985). Blackler & Fischberg (1961) found that these clusters are situated just anterior to the anus. When this region is grafted anteriorly to produce normal-pp larvae the clusters of germ cells are likely to be situated near the interface of graft- and posterior host endoderm, relatively close to the prospective genital ridges. This may imply that the signal from the posterior axial mesoderm which directs germ cell migration (Gipouloux, 1970) can reach the cluster of germ cells in the midendoderm of normal-pp larvae. But the signal apparently cannot reach most of the germ cells distributed throughout the anterior endoderm of inverted embryos.

In addition to this the possibility exists that germ cells in a cluster facilitate each other's migration. Individual germ cells show a tendency to take a route already taken by another and they have been observed to migrate in file (Delbos, Gipouloux & Guennon, 1982).

Effectiveness of grafting in inverted-aa larvae

The data obtained do not allow a more quantitative assessment of the effectiveness of grafting, i.e. did *all* the germ cells from the graft in inverted-aa larvae migrate to the genital ridges? Although the average number of germ cells was considerably higher in this group than in inverted embryos, they did not reach the same level as in controls (Table 2). Such an intermediate value is to be expected for two reasons. Firstly, rotation itself reduces the total number of germ cells by about 20 % (Thomas *et al.* 1983; Cleine & Dixon, 1985) and secondly, the anterior half of the endoderm of an inverted embryo, which is taken as the graft, does not contain the same high proportion of the embryo's germ cells as the posterior half of a normal larva (Cleine & Dixon, 1985).

A more exact analysis is further complicated by the fact that the anterior limit from where germ cells can migrate does not necessarily coincide with the vertical cut edge which separates anterior from posterior endoderm. As already indicated

by the results with normal-pp larvae germ cells anterior to the cut edge, but still close to it, can migrate successfully. So the germ cells in the genital ridges of inverted-aa larvae may in some cases include a few host germ cells as well. However, the majority of germ cells must be of graft origin.

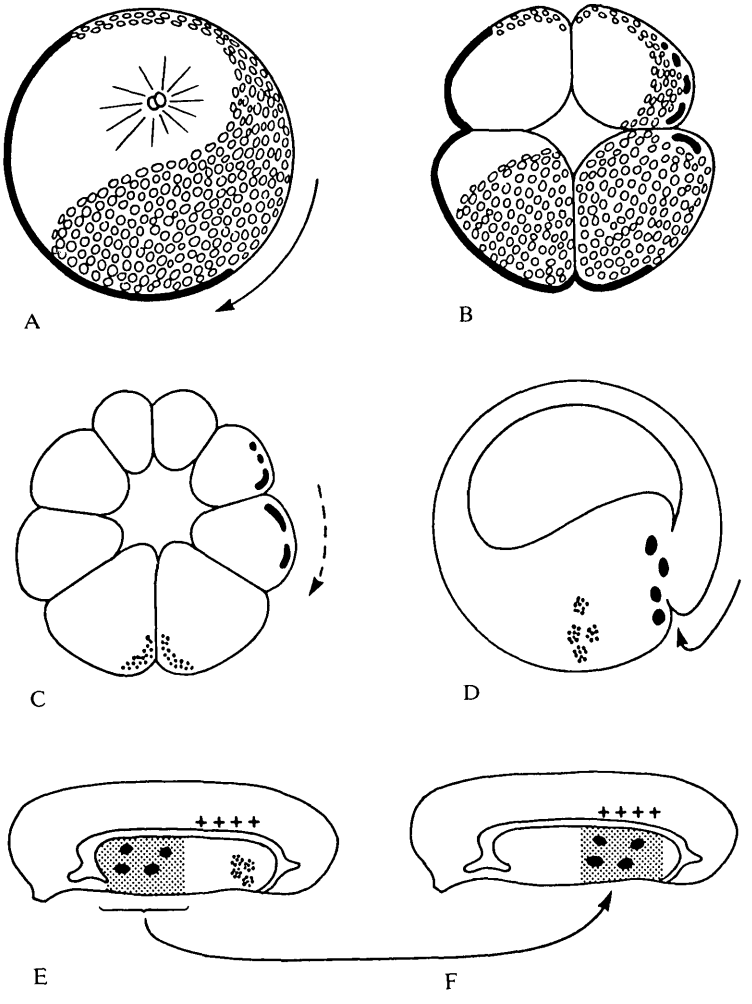

Fig. 4. Diagram of the events which lead to the relocation of germ cells in inverted embryos. Embryos are shown in the median plane. (A) 1-cell stage. The heavy yolk slips largely into the pigmented hemisphere (arrow). (B) 8-cell stage. Germ plasm (black) retains its original position opposite the pigment cap. (C) Morula. Germ plasm in dorsal equatorial blastomeres. Descendants of these blastomeres later undergo epiboly (arrow) (see Keller, 1978). In a comparable control germ plasm would be at the vegetal pole (dotted). (D) Onset of gastrulation. Cells containing germ plasm (black) near the dorsal lip. They invaginate further than those in controls (dotted). (E) Tailbud larva. Most germ cells localize in anterior endoderm (shaded) from where they cannot migrate to the presumptive genital ridges (++++). Germ cells in controls are in posterior endoderm (dotted). (F) Germ cells in graft can reach the genital ridges after transplantation to posterior region. (A–D: based on Cleine & Dixon, 1985).

The effect of grafting, together with the preceding events which lead to the relocation of germ cells in the anterior endoderm of inverted embryos are illustrated in Fig. 4. The results show that cells which inherit germ plasm, despite its ectopic position, keep the potential to differentiate as primordial germ cells. This is supportive evidence for the determinative role of germ plasm in germ cell differentiation.

Fate of ectopic germ cells

It is not known what happens to the germ cells in the anterior endoderm of inverted embryos. There is no delayed migration to the genital ridges (Cleine & Dixon, 1985). Germ cells that are unable to migrate out of the endoderm may degenerate at an early stage like those whose migration is prevented by PNA-lectin (Delbos, Gipouloux & Saidi, 1984). In tadpoles at stage 48 ectopic germ cells have occasionally been observed in anterior endodermal derivatives, notably in and on the epithelium of the oesophagus and possibly on the epithelium of the gut (Cleine, unpublished observations). This suggests that in inverted embryos at least some germ cells follow a highly disturbed route without being guided to the genital ridges. Recently it was reported that isolated primordial germ cells of *X. laevis*, after being inserted into a host embryo's blastocoel, were later located in the somites where they showed features of muscle cell differentiation (Heasman, 1985). This shows that ectopic primordial germ cells can still be responsive to environmental cues and that could imply that under normal conditions the environment may also be important for the late determinative events which lead to gametogenesis while germ plasm might then have a lesser role. It is known from u.v.-irradiation experiments that germ plasm is indispensable for the early events in the germ cell lineage, notably germ cell migration (Smith & Williams, 1979; Thomas *et al.* 1983; Subtelny & Penkala, 1984; Williams & Smith, 1984). However, the precise role of germ plasm and other factors in each step of germ cell differentiation as well as the establishment of their molecular identity awaits further analysis.

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