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Phil. Trans. R. Soc. Lond. B 1985 **312**, 145-152
doi: 10.1098/rstb.1985.0184

Phil. Trans. R. Soc. Lond. B **312**, 145–152 (1985) [145]
Printed in Great Britain

Cell lineage and commitment in early amphibian development

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[Plates 1 and 2]

It is clear by the neurula stage of amphibian development that the three primary cell types or germ layers can be recognized. However, little is known about when and how individual cells of the blastula and gastrula become committed to these germ layers.

The laser dye tetramethyl rhodamine isothiocyanate is proving a useful cell marker for studies of cell commitment. When labelled cells from the animal or vegetal pole of *Xenopus laevis* early embryos are transplanted into host blastocoels, their progeny become specified to different germ layers and differentiate in accordance with surrounding cells. By using this method we have shown that animal and vegetal pole cells are pluripotent at the early blastula stage, while vegetal pole cells are committed to form endoderm by the early gastrula stage of development. One population of cells, the primordial germ cells, which arise from the vegetal part of the early embryo, are shown still to be pluripotent at the swimming tadpole stage.

1. Introduction

Classical studies on amphibian embryos have made it clear that the three primary cell types, the germ layers, ectoderm, mesoderm and endoderm can be recognized in sectioned material by the neurula stage of development (Spemann 1938). Furthermore, explant experiments (Holtfretter 1925; Spemann 1938; Sudarwati & Nieuwkoop 1971; Nakamura 1978; Gurdon *et al.* 1984) have shown that, from the mid blastula stage onwards, at least some areas of the embryo have a capacity for normal self-differentiation into the appropriate germ layer.

One conspicuous gap in our knowledge however, is in understanding when and how individual cells in the early embryo become restricted to particular cell lineages. One reason for this is that it is only recently that appropriate and reliable cell lineage markers have become available. This review focuses on our recent studies on this problem of cell commitment. It includes a description of the single cell labelling technique used, a brief account of our operational definition of commitment, and reviews the application of this method to test the developmental capacity of individual cells from different parts of *Xenopus laevis* embryos.

2. The method of single cell labelling

For these experiments we have used the laser dye tetramethyl rhodamine isothiocyanate (TRITC) as a vital stain (Heasman *et al.* 1984). Initially we tested the suitability of TRITC as a cell marker by using it to stain primordial germ cells which we collected from *Xenopus* larvae (Wylie & Roos 1976). Isolated primordial germ cells were labelled by immersion in 70% Liebovitz medium containing 10% foetal calf serum, containing the dye at a concentration of 200 μg ml^{-1}, (TRITC stock solution is made up in 2% $NaHCO_3$). The primordial germ

cells were placed on a substrate of epithelial cells derived from *Xenopus* mesentery for three days. They attached, spread and behaved in all respects like unlabelled germ cells (figure 1, plate 1). When the culture was viewed under ultraviolet light by using rhodamine excitation filters, we found that the label was not transferred to other cells (since cytological criteria establish the labelled cells as germ cells), even though we know primordial germ cells form junctions with surrounding cells. From this preliminary work we concluded that TRITC is a useful cell marker fulfilling the following criteria: it does not interfere with normal cell behaviour, does not spread to adjacent, unlabelled cells and is visible in labelled cells for at least three days. The label can be made visible easily and is not destroyed by fixatives such as paraformaldehyde, glutaraldehyde or trichloracetic acid, or embedding media such as gelatin or wax.

We therefore adopted this method of cell labelling for studies on the state of commitment of single cells of *Xenopus laevis* embryos. Three further controls were required for this system. Firstly, we confirmed that TRITC does not interfere with the normal division rate of isolated *Xenopus* blastomeres (Heasman *et al.* 1984). Secondly, it seemed important to test that rhodamine is cell-autonomous when used in this system and does not diffuse into surrounding cells. We tested this in three different ways (Heasman *et al.* 1984). For example, we labelled vegetal pole cells from *Xenopus borealis* with TRITC and implanted them into embryos of *Xenopus laevis*. The progeny of implanted cells were identified by both TRITC labelling and a monoclonal antibody directed against *Xenopus borealis* nuclear antigen (courtesy of P. Hausen and C. Dreyer), by using fluorescein conjugated second antibody. When several hundred cells were examined, none were labelled by one technique and not the other.

Finally we needed to test whether the progeny of isolated labelled blastomeres could take part in normal development and differentiate. These experiments are described below.

3. The experimental design for testing the state of commitment of individual cells of *Xenopus* blastulae and gastrulae

Classical embryologists (Vogt 1929) used vital stains to label parts of the embryo surface and follow the fate of each area during development. From such data, fate maps were constructed which, because there is little random cell mixing during early embryogenesis, allow us to predict which germ layer each group of cells or rather, their progeny, will eventually occupy. Recently fate-mapping experiments have been repeated more accurately and revised maps are now available for both superficial and deep cells of the early embryonic stages (Keller 1975, 1976; Nakamura 1978; Hirose & Jacobson 1979; Smith & Slack 1983). The importance of this work for studies on cell commitment is that we can choose particular areas of the blastula and, knowing their prospective fate, ask the question, when during early development do those cells or their progeny lose the ability to enter any other cell lineage, that is, when do they become restricted and committed to their normal fate?

The operational test we have used to define the state of commitment of blastomeres is to isolate single cells from particular regions of *Xenopus* blastulae and gastrulae, label them with TRITC (50–100 μg ml^{-1}) and then to transplant them individually into the blastocoels of late blastula hosts (figure 2). In most experiments, the host embryos were allowed to develop to the late tailbud stage (stage 36, Nieuwkoop & Faber 1956), at which time the three germ layers can be distinguished clearly. Each normal host was then fixed in 2% TCA, embedded in low temperature wax and serially sectioned at 10 or 15 μm. Each section was examined under

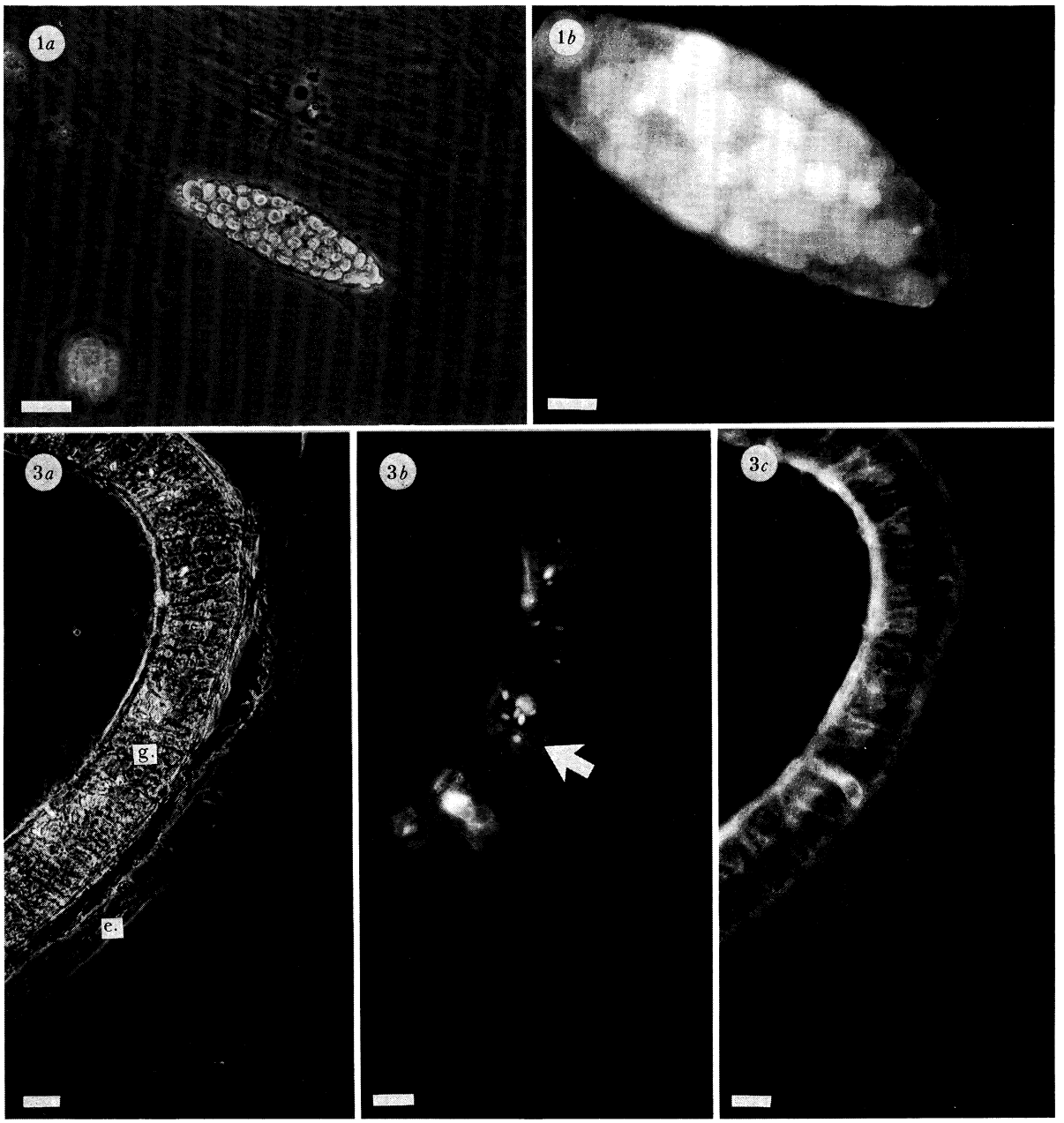

FIGURE 1. (*a*) Phase contrast picture of a primordial germ cell on a monolayer of cells derived from *Xenopus* mesentery. The primordial germ cell is distinctive because of its elongated shape and cytoplasm packed with yolk platelets. It was labelled with TRITC before culture. Scale bar, 10 μm. (*b*) One end of the same primordial germ cell as seen in (*a*), viewed under ultraviolet light by using rhodamine excitation filters. The primordial germ cell is brightly fluorescent while surrounding cells are completely unlabelled. Scale bar, 2.5 μm.

FIGURE 3. A test of the state of differentiation of the progeny of a TRITC-labelled animal pole cell. All three figures show the same section of part of a loop of gut and body wall of a stage 46 embryo. (*a*) A phase contrast picture to show the columnar nature and brush border of the gut epithelium. (*b*) A fluorescence picture with the rhodamine excitation filter making visible the labelled progeny in the gut. (*c*) A fluorescence picture with fluorescein excitation filters showing staining with VC1, a monoclonal antibody directed against endoderm cells of *Xenopus* embryos. Scale bar, 20 μm. g., Gut epithelium; e., epidermis.

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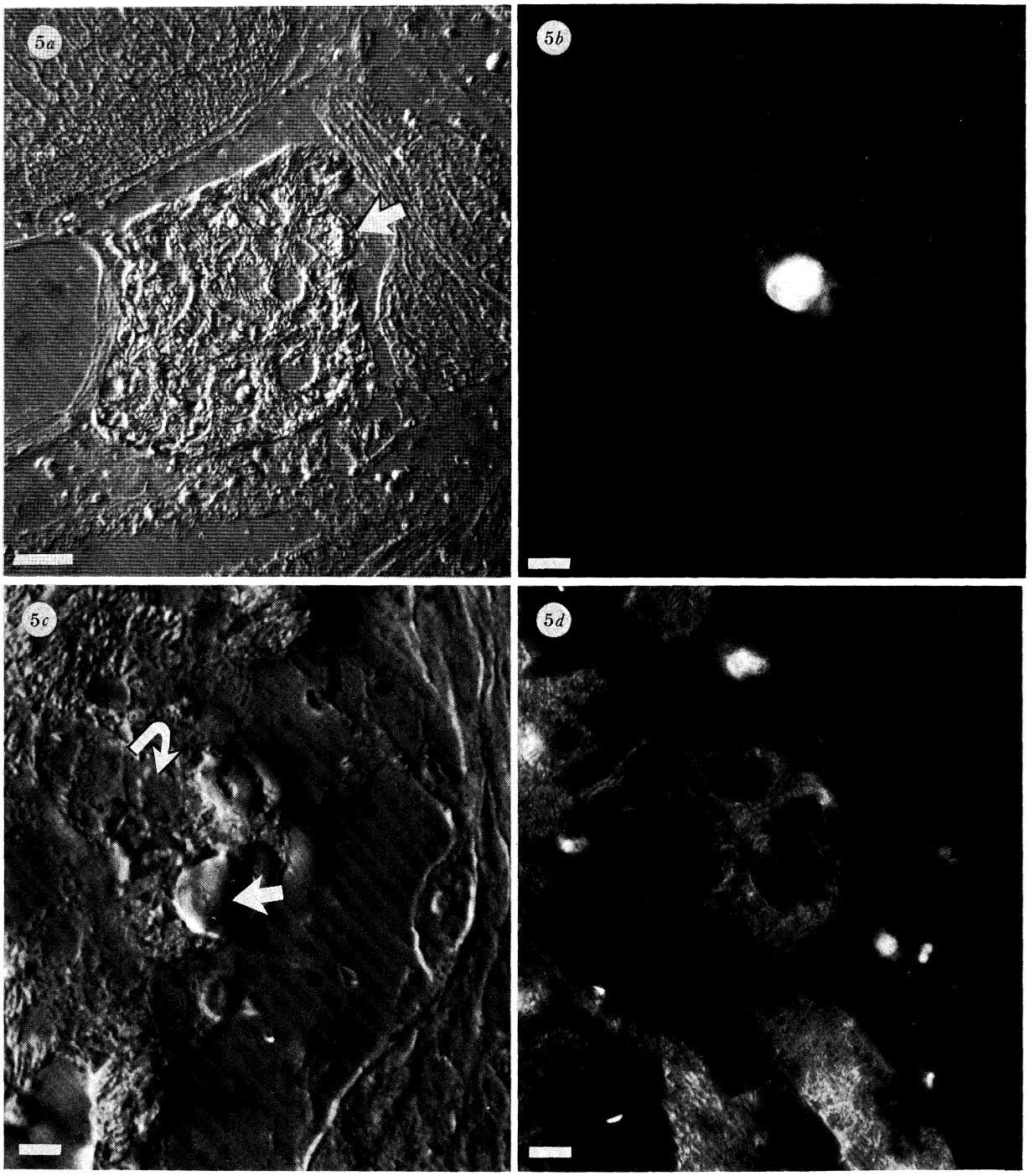

FIGURE 5. The result of transferring TRITC-labelled primordial germ cells into the blastocoel of a late blastula. (*a*)–(*d*) A TRITC-labelled cell in developing striated muscle of the myotome. (*a*) A low power Nomarski image showing the labelled cell (arrowed) in the lateral border of the myotome; (*b*) the TRITC labelling of the cell arrowed in (*a*). (*c*), (*d*) The next section (10 μm) of the same cell: (*c*) is a high power Nomarski image showing the cell's yolk platelets (straight arrow) and lobed nucleus (curved arrow); (*d*) shows form birefringence in the cytoplasm of the labelled cell, in common with the other cells of the myotome. Host primordial germ cells (unlabelled) in the developing gonadal ridge do not show form birefringence. Scale bars: (*a*), 50 μm; (*b*), 25 μm; (*c*), 5 μm; (*d*), 5 μm. (Wylie *et al.* 1985*b*).

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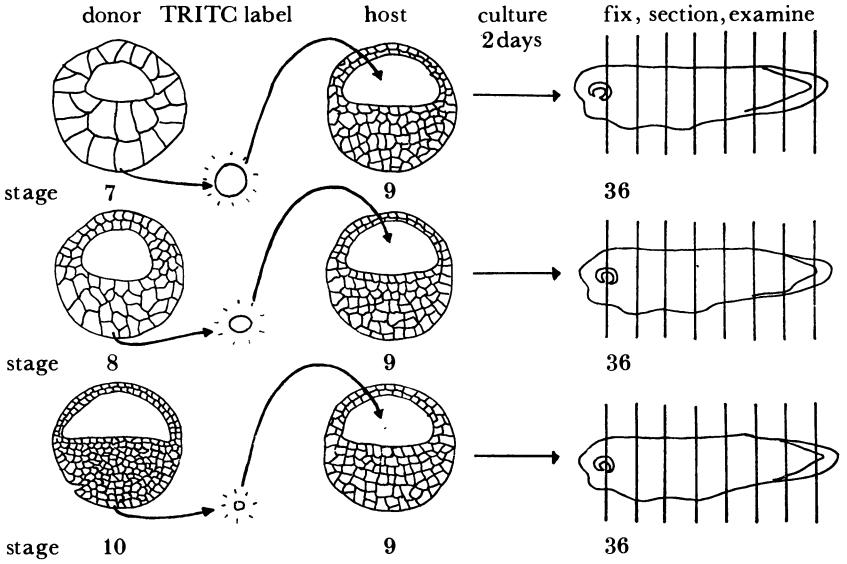

FIGURE 2. The design of the experiment to test the state of commitment of blastomeres. In this example, individual cells were isolated from the vegetal poles of blastulae and gastrulae, labelled with TRITC, and one labelled cell was placed in each host blastocoel.

ultraviolet light for labelled progeny of the original donor cell. If the progeny of the donor cell were found in all three germ layers, we considered the donor cell was pluripotent at the time of isolation. If they were restricted to one germ layer we assumed that the donor cell was committed to that germ layer at the time of isolation.

One possible criticism of this operational definition of commitment is that labelled cells might simply become trapped in ectopic germ layers after being placed in the blastocoel. If this were the case, then we would expect progeny not to take part in normal development and not to differentiate in accordance with their surroundings. To test this possibility we have allowed hosts to develop to the swimming tadpole stage, by which time overt differentiation of the three germ layers has occurred. We find that, in many cases, labelled progeny could be clearly seen to be differentiating in concert with their environment. Figure 3 shows a group of labelled progeny from a cell isolated from the animal pole of a mid blastula stage embryo. The normal fate of cells from this region is to form ectodermal derivatives. However, these progeny are found in the wall of a loop of gut. The cells are morphologically similar to other gut epithelial cells, often having a columnar shape and a brush border (see arrow on figure 3*b*). Furthermore, the labelled cells stain with an antibody (VC1) which is almost specific for gut endoderm in *Xenopus* embryos (Wylie *et al.* 1985*a*). Labelled progeny situated in mesoderm and ectoderm do not stain with this antibody.

These data show that the operational test of commitment that we use is a sound one. When a cell is injected into a host blastocoel, it is provided with an environment which allows the whole range of capabilities of the cell to be tested. Thus a pluripotent cell gives progeny in all germ layers and those progeny differentiate in accordance with the area in which they lie. Cells do not become 'trapped' and unable to differentiate although, without a complete range of differentiation markers, it is not possible to verify that they always acquire an appropriate phenotype.

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4. The states of commitment of cells from different areas of *Xenopus* embryos

We have applied the technique described above to the study of cells from different areas of *Xenopus* embryos. The aim has been firstly to pinpoint the time at which cells become restricted to particular cell lineages, and secondly to study the mechanism of this restriction.

(*a*) *Vegetal pole cells*

The normal fate of cells of the vegetal pole of *Xenopus* blastulae is to form the gut endoderm (Keller 1975, 1976; Heasman *et al.* 1984). We tested when cells become restricted to this fate by taking blastomeres from this region of early, mid and late blastula and early gastrula stages, and transplanting them into the blastocoels of late blastulae hosts (figure 2). We find that while early blastula cells from this region are pluripotent, the vegetal pole cells become completely committed to form endoderm only by the early gastrula stage (figure 4).

Interestingly, the results show that commitment to endoderm is a gradual process, and is not the result of a single rapid switch occurring in all cells in the vegetal area at the same time. The sequence of steps involved in this process is not clear, but they can be approached by studying in more detail the 'partly committed states', that is, the mid blastula, in which 40 %

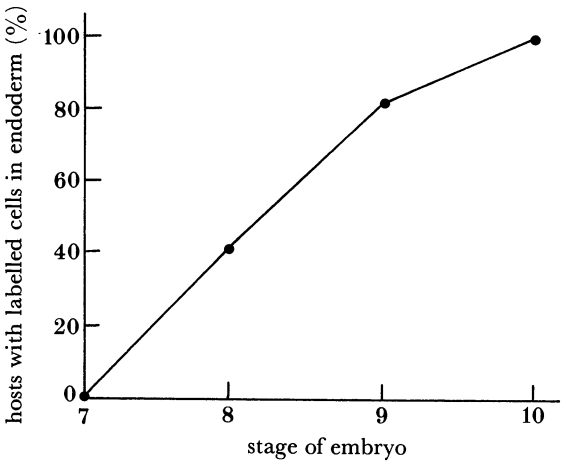

Figure 4. Embryonic stage and the state of commitment to endoderm of vegetal pole cells, using the test of commitment described in figure 2.

Table 1. Compiled results of superficial vegetal pole cell transfers

source of donor cells	number of hosts with labelled cells	The number of hosts containing labelled cells in the following combinations of germ layers						
		end mes ect	end only	end mes only	end ect only	mes ect only	mes only	ect only
st. 7 early blastula	8	8	0	0	0	0	0	0
st. 8 mid blastula	66	19	26	17	1	3	0	0
st. 9 late blastula	67	2	55	8	0	0	2	0
st. 10 early gastrula	22	0	22	0	0	0	0	0

of transferred cells have progeny only in endoderm, and the late blastula, where 82% of labelled donor cells have progeny only in endoderm. These experiments are in progress.

(*b*) *Animal pole cells*

The fate maps of *Xenopus* late blastulae and early gastrulae show that the animal pole region becomes ectodermal derivatives (Keller 1975, 1976). To find out when cells from this region become restricted to this fate, we have carried out experiments similar to those described above, using animal pole donor cells.

We took cells from the early, mid and late blastula, labelled them and injected them into the blastocoel cavities of host embryos. In preliminary experiments, we used hosts of the late blastula stage as before. More recently we have altered the protocol by using later stage host embryos (stage 10–11, early and mid gastrula stages). The reason for this is that we found that the blastocoel cavity of the late blastula is not a neutral environment for animal pole cells, but actually restricts their range of developmental potential (A. Snape, J. Heasman and C. C. Wylie, unpublished observations). This was suggested by the earlier experiments of Nieuwkoop & Ubbels (1972) and Nakamura (1978). They showed that when pieces of tissue derived from the animal pole of *Xenopus* blastulae are cultured with the vegetal mass of a mid blastula, the animal pole cells are 'vegetalized' to form endoderm and mesoderm. As, in our experiments, most injected cells fall to the floor of the host blastocoel and therefore onto the vegetal mass, we might expect the vegetal cells to influence the transplanted donors.

TABLE 2. RESULTS OF TRANSFERS OF STAGE 7 ANIMAL POLE CELLS INTO HOSTS OF DIFFERENT STAGES

		The number of hosts containing labelled cells in the following combinations of germ layers						
host age at time of transfer	number of hosts with labelled cells	end mes ect	end only	end mes only	end ect only	mes ect only	mes only	ect only
stage 8 hosts	12	11	0	1	0	0	0	0
stage 9/10 hosts	16	13	2	0	1	0	0	0

TABLE 3. RESULTS OF TRANSFERS OF STAGE 9 ANIMAL POLE CELLS INTO HOSTS OF DIFFERENT STAGES

		The number of hosts containing labelled cells in the following combinations of germ layers						
host age at time of transfer	number of hosts with labelled cells	end mes ect	end only	end mes only	end ect only	mes ect only	mes only	ect only
stage 8 hosts	18	3	11	2	1	1	0	0
stage 11	29	1	4	3	4	7	0	10

To test this we placed early and late blastula animal pole cells into 'vegetalizing' (stage 8) and non-vegetalizing (stage 10 and 11) hosts. The results are summarized in tables 2 and 3. Two interesting findings emerge from this work: first, we found that early blastula cells are pluripotent and their progeny are found in all germ layers, whatever the host age. Furthermore, they differentiate according to their site in the embryo. We can conclude that, according to

our definition of commitment, animal pole cells as well as those of the vegetal pole of the early blastula are pluripotent. Second, we find that the age of the host does influence the developmental potential of mid and late blastula animal pole cells. In earlier, vegetalizing hosts, these cells give progeny in a variety of tissues, particularly in mesoderm and endoderm derivatives and not in ectoderm only. In contrast, when later, non-vegetalizing hosts are used, 30% of labelled donor cells give progeny only in ectoderm. This suggests that the donor cells are in a 'partly committed' state at the late blastula stage. This is a labile and reversible situation wherby animal pole cells can be induced by a vegetalizing host to form endoderm or mesoderm. We are currently studying animal pole cells from later embryos to pinpoint when cells from the region become committed to form ectoderm only.

(*c*) *Primordial germ cells*

From the work described above, we can conclude that at both the animal and vegetal poles there is a gradual restriction of potential for cells and their progeny, resulting in germ layer commitment during gastrula stages. It has generally been assumed that in amphibia, the germ cell lineage also becomes specified during these early embryonic stages (for reviews see Bounoure 1939; Smith & Williams 1979). Classical studies suggest that presumptive primordial germ cells arise at the vegetal pole and contain a cytoplasmic determinant, 'germ plasm', inherited from the unfertilized egg. We wanted to test whether primordial germ cells are indeed committed to the germ cell lineage. We isolated primordial germ cells from the swimming tadpole stage embryo, when they are in their final stages of migrating into the gonad. They were labelled and transplanted into the blastocoel cavity of late blastula hosts (Wylie *et al.* 1985*b*). This was not a single cell analysis as approximately 15 primordial germ cells were injected into each host.

We found that in 14 hosts containing labelled cells, the primordial germ cells were able to participate in development after insertion into the blastocoel. Furthermore, labelled cells were

TABLE 4. GERM LAYER DERIVATIVES CONTAINING LABELLED CELLS AFTER INJECTION OF TRITC-LABELLED PRIMORDIAL GERM CELLS AT THE LATE BLASTULA STAGE (WYLIE *ET AL.* 1985*b*)

embryo number	number of labelled cells in ectoderm	mesoderm	endoderm	total number of labelled cells
1	5	0	1	6
2	5	2	0	7
3	3	1	0	4
4	6	1	2	9
5	2	1	0	3
6	0	1	1	2
7	0	1	3	4
8	0	0	7	7
9	2	3	0	5
10	0	0	0	0
11	1	1	0	2
12	0	0	0	0
13	0	0	0	0
14	4	1	2	7
15	0	0	1	1
16	3	2	0	5
totals	**31**	**14**	**17**	**62**

found in all germ layers and were morphologically differentiated in accordance with these sites, while retaining their distinctive nuclear morphology (table 4 and figure 5). In particular, no labelled primordial germ cells were found in the gonadal primordia of the host embryos at the swimming tadpole stage. The conclusion from these data is that primordial germ cells are not committed to enter the germ line at the time when they are migrating to the gonadal ridge (Wylie *et al.* 1985). In contrast, other cells that originate in the vegetal pole of the early embryo become committed by the early gastrula stage to form endoderm only. It will be interesting to test later germ-line cells such as oogonia and spermatogonia in the same way to discover when germ-line cells become committed to their normal fate.

5. Is cell size important in commitment?

As early *Xenopus* embryos develop they do not grow. Consequently, cells isolated from progressively later stages of blastulae and gastrulae are progressively smaller in size. It is possible that these size differences influence the developmental capacity of labelled blastomeres when we place them into the blastocoel cavity of host embryos. Thus large cells of the early blastula almost fill the blastocoel cavity, and might come into contact with cells destined to form all three germ layers, while small early gastrula cells fall to the floor of the blastocoel and therefore may be influenced only by the vegetal mass.

We think that this rather trivial explanation of the results is not the case for a number of reasons. First, animal pole cells taken from early blastulae are the same size as vegetal pole cells taken from the mid blastula stage. However, the two groups of cells have different states of commitment. Animal pole cells are pluripotent while 40% of vegetal pole cells give progeny in endoderm only.

Second, experiments on the vegetal pole, in which cells from the mid blastula were cultured until sibling embryos reached the early gastrula stage, showed that the cells *in vitro* divided at the same rate as those in the embryo. Thus isolated and cultured mid blastula cells form clones of the same size as freshly collected early gastrula cells. However, the developmental capacity of the two groups of cells is different when they are transplanted into host blastocoels. The progeny of the cultured cells remain only 40% committed to form endoderm only, while the newly isolated cells are 100% committed to endoderm (see Heasman *et al.* (1985) for more details).

Finally, primordial germ cells are smaller than both animal and vegetal blastomeres and yet they are not committed to one germ layer. For these reasons, it seems unlikely that cell size affects the range of cell lineages available to embryonic cells placed in host blastocoels.

6. Conclusions

Transplantation experiments of the type described here, using TRITC as a cell marker, provide a useful method for testing the developmental capacity of embryonic amphibian cells. The method could easily be adapted to other embryonic systems in which no growth occurs during early development (for example, fish, urodele, invertebrate embryos). However, TRITC would only be a useful marker for short term experiments in chick and mammalian systems. Ziomek & Johnson (1982) have used a similar dye, fluorescein isothiocyanate, to label cells of early mammalian embryos for a 24 h period of culture.

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The results described here show that during the blastula and early gastrula stages cells become committed to ectoderm and endoderm cell types. The cells of the early blastula are pluripotent and at least one population of cells, the primordial germ cells, retain a wide developmental capacity even until the swimming tadpole stage. The mechanisms governing these processes are still far from being understood. However, we are now in a position to analyse, at a cellular and molecular level, the differences between committed and non-committed populations of cells and, by using the transplantation system described here, test the developmental significance of these differences experimentally.

The authors thank the Wellcome Trust and the Cancer Research Campaign for their support. Excellent technical assistance was provided by Miss Claire Varley and Mrs Lynn Albert. Thanks also to Melanie Coulton for secretarial assistance.

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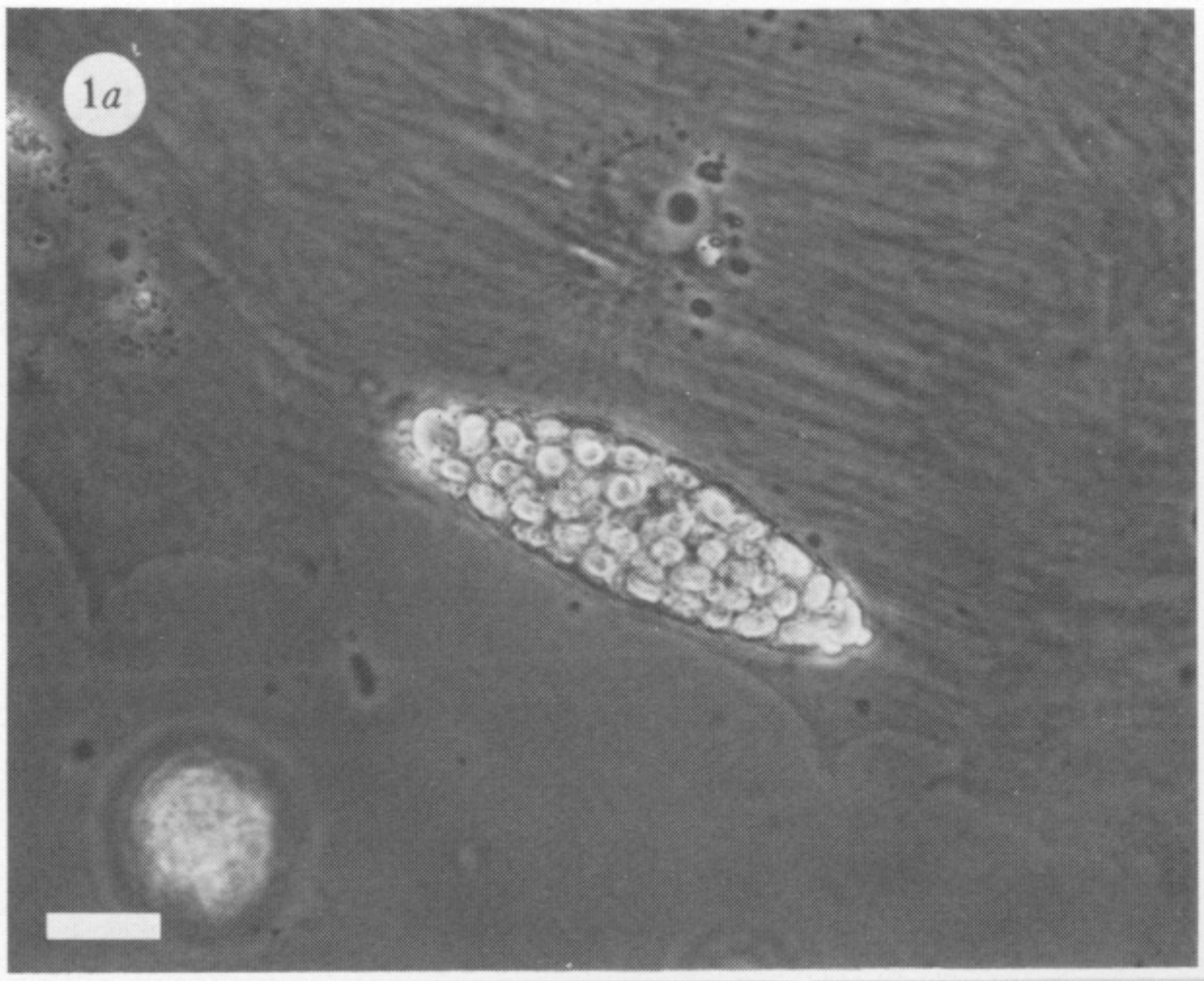

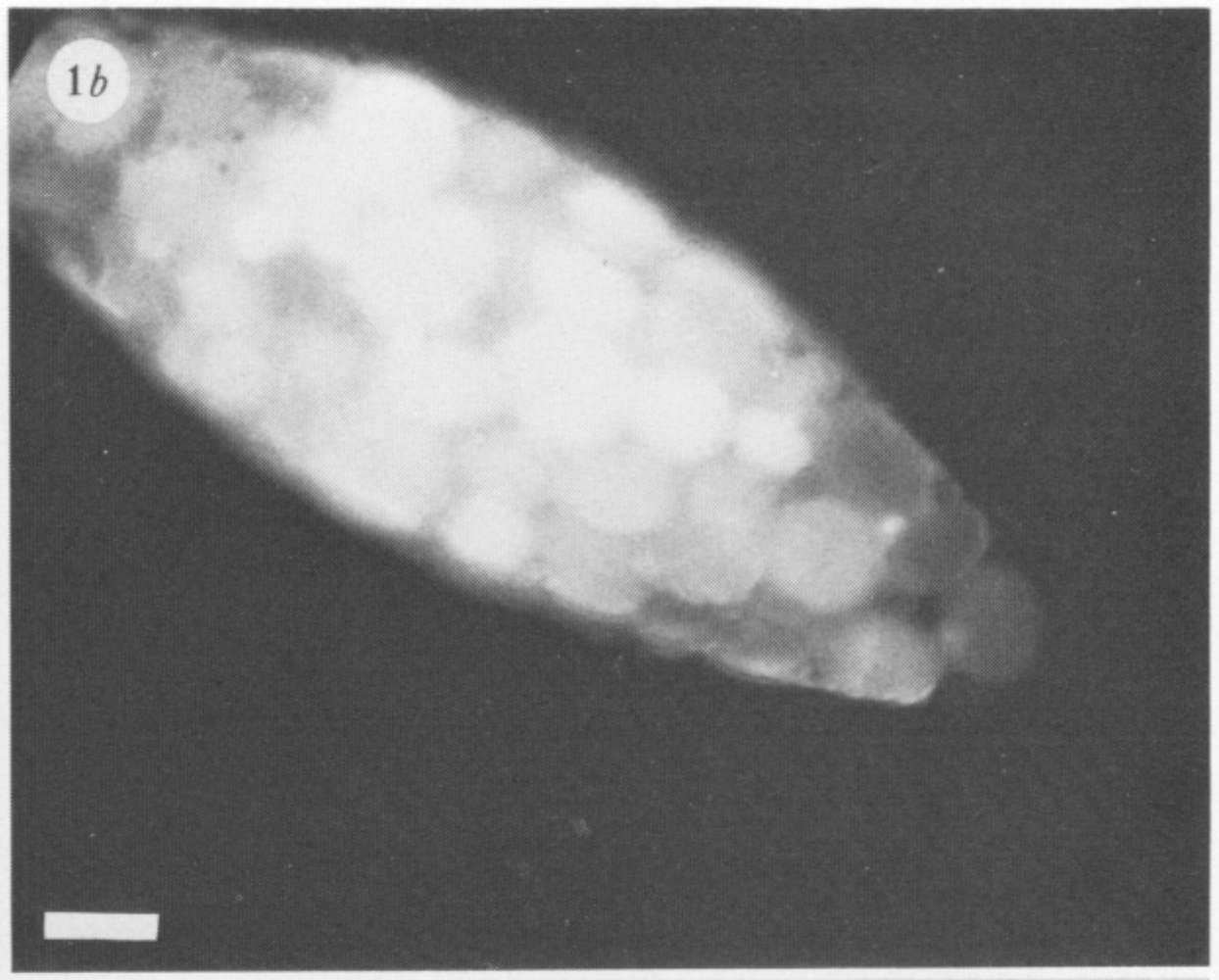

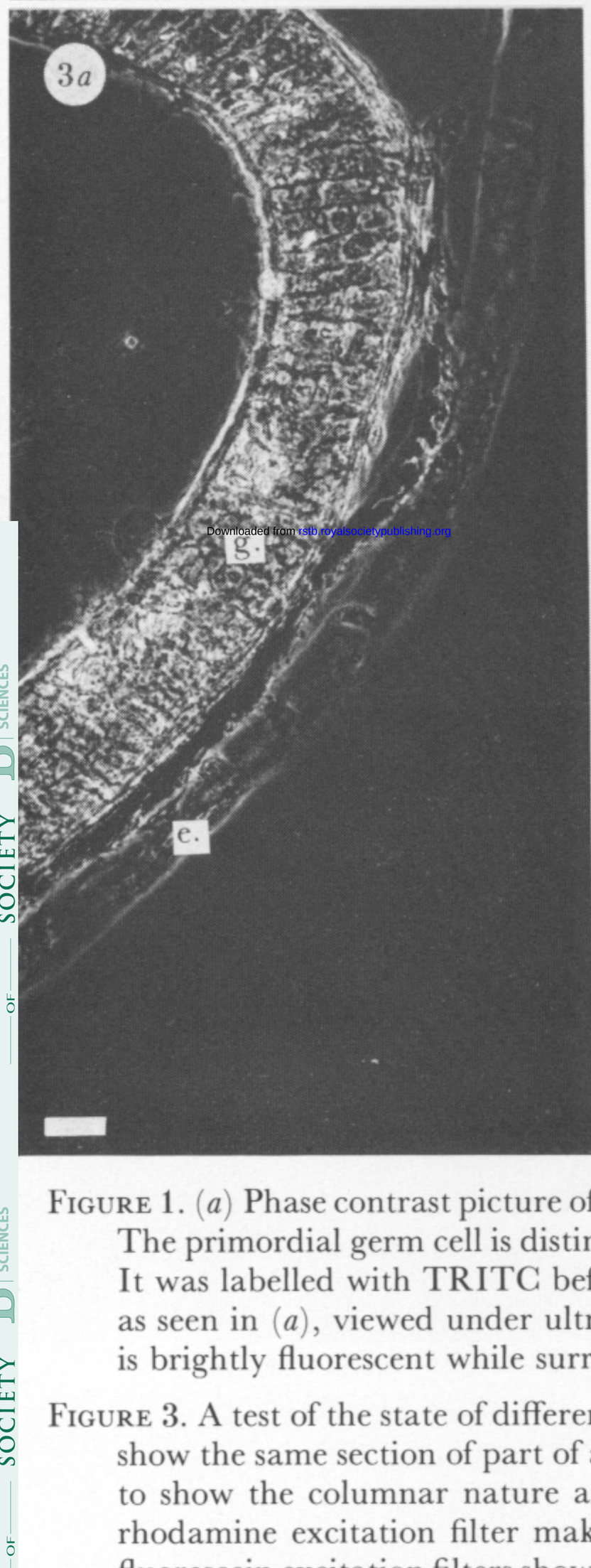

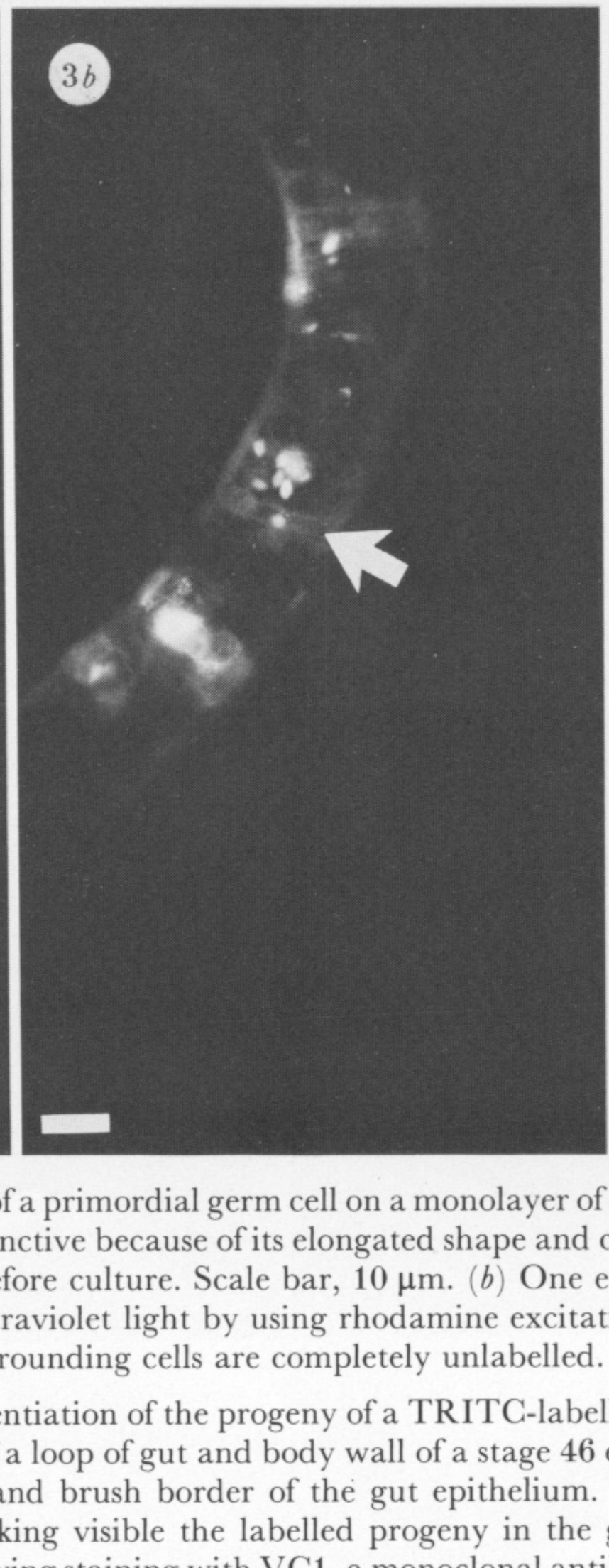

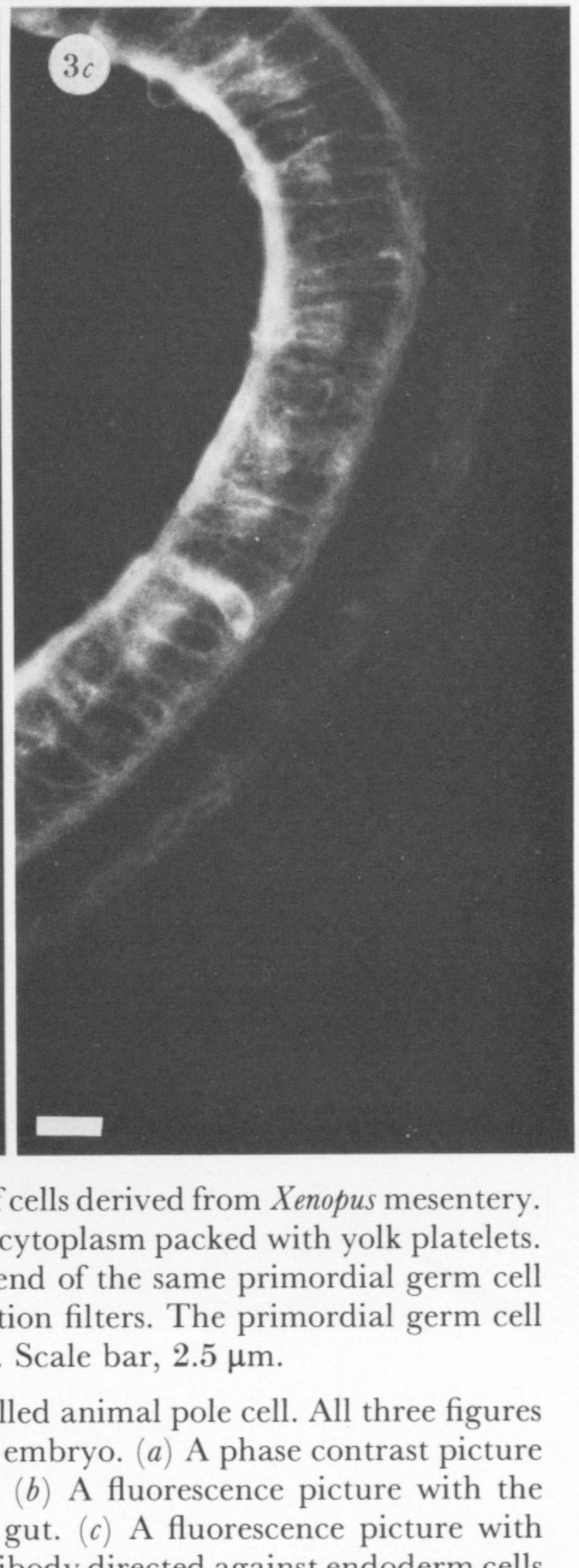

FIGURE 1. (*a*) Phase contrast picture of a primordial germ cell on a monolayer of cells derived from *Xenopus* mesentery. The primordial germ cell is distinctive because of its elongated shape and cytoplasm packed with yolk platelets. It was labelled with TRITC before culture. Scale bar, 10 μm. (*b*) One end of the same primordial germ cell as seen in (*a*), viewed under ultraviolet light by using rhodamine excitation filters. The primordial germ cell is brightly fluorescent while surrounding cells are completely unlabelled. Scale bar, 2.5 μm.

FIGURE 3. A test of the state of differentiation of the progeny of a TRITC-labelled animal pole cell. All three figures show the same section of part of a loop of gut and body wall of a stage 46 embryo. (*a*) A phase contrast picture to show the columnar nature and brush border of the gut epithelium. (*b*) A fluorescence picture with the rhodamine excitation filter making visible the labelled progeny in the gut. (*c*) A fluorescence picture with fluorescein excitation filters showing staining with VC1, a monoclonal antibody directed against endoderm cells of *Xenopus* embryos. Scale bar, 20 μm. g., Gut epithelium; e., epidermis.

FIGURE 5. The result of transferring TRITC-labelled primordial germ cells into the blastocoel of a late blastula. (*a*)–(*d*) A TRITC-labelled cell in developing striated muscle of the myotome. (*a*) A low power Nomarski image showing the labelled cell (arrowed) in the lateral border of the myotome; (*b*) the TRITC labelling of the cell arrowed in (*a*). (*c*), (*d*) The next section (10 μm) of the same cell: (*c*) is a high power Nomarski image showing the cell's yolk platelets (straight arrow) and lobed nucleus (curved arrow); (*d*) shows form birefringence in the cytoplasm of the labelled cell, in common with the other cells of the myotome. Host primordial germ cells (unlabelled) in the developing gonadal ridge do not show form birefringence. Scale bars: (*a*), 50 μm; (*b*), 25 μm; (*c*), 5 μm; (*d*), 5 μm. (Wylie *et al.* 1985*b*).