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Spreading of microinjected horseradish peroxidase to nondescendant cells in embryos of *Patella* (Mollusca, Gastropoda)

WIEL M. KÜHTREIBER, FLORENCI SERRAS and JO A. M. van den BIGGELAAR

Department of Experimental Zoology, University of Utrecht, Padualaan 8, 3584 CH Utrecht, The Netherlands

Summary

We have injected horseradish peroxidase (HRP) and fluorescein–isothiocyanate dextran (FD) into cells and into the blastocoelic cavity of *Patella vulgata* embryos, before and during the interval between 5th and 6th cleavage, in which the mesodermal stem cell is determined by means of interactions between the central 3D macromere and the contacting animal micromeres.

Intracellular injections of HRP at different stages showed that, whereas before this contact phase no spreading of label was observed, a clear intercellular transfer of HRP was found after the contact was established. Control experiments showed that it was HRP in its intact, high molecular weight form that was transferred in the living embryo.

Injections of HRP into the blastocoelic cavity gave essentially the same results. In these cases, the HRP was taken up by the cells from contact stage onwards. When FD was injected into the blastocoelic cavity, no uptake was observed, not even after prolonged presence of FD in it. However, when HRP and FD were mixed, both were taken up, starting at contact stage. Differences in labelling pattern of HRP, as compared with FD, and a shift of the FD fluorescence after uptake, suggest that receptor-mediated endocytosis is involved. The possible morphogenetic significance of the transfer mechanism is discussed.

Key words: intercellular communication, horseradish peroxidase, endocytosis, *Patella*, HRP, fluorescein–isothiocyanate dextran.

Introduction

In embryos of equal cleaving molluscs like *Patella*, the mesodermal stem cell is determined during the interval between 5th and 6th cleavage. Out of four possible candidates (i.e. the four macromeres), only the macromere that contacts the animal micromeres will be induced (van den Biggelaar, 1977; van den Biggelaar & Guerrier, 1979; van den Biggelaar, Dorresteijn, de Laat & Bluemink, 1981). The mechanisms of the cellular interactions involved in this inductive process during the contact phase are still unknown.

Three main mechanisms are supposed to be involved in induction in general (Slack, 1983): (1) exchange of diffusible molecules over relatively long distances, (2) exchange of signal molecules *via* intercellular communication channels (Hooper & Subak-Sharpe, 1981; Finbow, 1982; Caveney, 1985) and (3) direct or indirect cell-to-cell contact for ligand–receptor coupling (Kühtreiber *et al.* 1986). Exchange of diffusible molecules over a relatively long distance does not appear to play a role, as in this case more than one macromere would be induced. This, however, does not occur (van den Biggelaar, 1977; van den Biggelaar & Guerrier, 1979). The second and third possibility may be of importance in the *Patella* embryo.

In *Patella* embryos, gap junctions are present from the 4-cell stage onwards (Dorresteijn, Bilinski, van den Biggelaar & Bluemink, 1982). However, no dye coupling is found until the 32-cell stage (Dorresteijn, Wagemaker, de Laat & van den Biggelaar, 1983). Although, after the 5th cleavage, all cells bordering each other along the periphery of the embryo are dye coupled, no direct dye coupling between the presumptive mesodermal stem cell (3D) and the overlying micromeres could be observed. From these experiments on the spreading of low molecular weight dyes, no definite conclusions could be drawn about the type of communication channel by which they were transferred. The most obvious candidates are gap junctions, which allow the passage of molecules up to a relative molecular mass (M_r) of about

1200 (Simpson, Rose & Loewenstein, 1977). In order to obtain additional information about intercellular channels and other possible transfer mechanisms that may exist in a 32-cell stage *Patella* embryo, we decided to label cells with the enzyme horseradish peroxidase (HRP, M_r approx. 40×10^3).

HRP can be easily detected and has been used as a tracer in a number of cell lineage studies in different developing systems (Weisblat, Sawyer & Stent, 1978; Nishida & Satoh, 1983; Goodall & Johnson, 1984; Weisblat & Blair, 1984; Weisblat, Kim & Stent, 1984; Cruz & Pederson, 1985; Nishida & Satoh, 1985). In these studies, HRP did not spread from the labelled cell to other blastomeres. During the successive stages, it could therefore only be detected in the progeny of the impaled cell. However, in our experiments, the HRP did spread from impaled cells to other blastomeres. Therefore, we have to assume the presence of an additional mechanism, besides gap junctions, for intercellular transfer in 32-cell-stage *Patella* embryos.

Materials and methods

Embryos

Adult specimens of the marine gastropods *Patella vulgata* and *P. coerulea* were collected in Roscoff or Dieppe (France) and Arenys de Mar (Spain), respectively. They were kept at 15°C in tanks with recirculating filtered seawater. Artificial fertilization was performed as described by van den Biggelaar (1977). Embryos of the appropriate stage were selected. To facilitate the intracellular injections, the embryos were washed in acidified seawater (pH 4·0) for 2–3 min to remove the gelatinous egg capsule which develops after the chorion has been stripped off. Embryos treated in this way continued to develop normally. All subsequent injection experiments were carried out at 18°C in Millipore-filtered seawater.

Microinjections

(1) *Iontophoresis*

Microelectrodes (tip diameter smaller than 0·1 μm, tip resistance <100 MΩ when filled with 3 M-KCl) were pulled from glass capillaries with inner filament (Clark Electromedical Instruments GC150F-15), and back-filled with a 3% solution of HRP (type II or VI, Sigma Chemical Company) or FITC–dextran (FD, M_r 41×10^3, Sigma) in 0·1 M-KCl. Iontophoresis was carried out essentially as described by Dorresteijn *et al.* (1983) with some modifications. HRP or FD was introduced into a cell using repetitive depolarizing pulses of 10 nA amplitude and 0·4 s duration at intervals of 3–4 s for about 7 min, or, alternatively, using four or five long pulses of 8–9 nA amplitude for about 10 s each at intervals of 10–20 s.

(2) *Pressure injections*

Direct (pressure) injections into cells or into the blastocoelic cavity were performed with a thermal expansion pressure system, which is described in detail elsewhere (Kühtreiber & Serras, 1987).

For intracellular injections, micropipettes with outer tip diameters smaller than 0·1 μm (i.e. below the resolving power of the light microscope) were used. The tracers (HRP, FD or mixtures of HRP and FD) were dissolved in 0·05 M-KCl or bidistilled water. Both solvents gave the same results and the embryos developed normally. The injected amounts varied between 0·5 and 2 pl, as measured by performing comparable injections into paraffin oil and determining the volume of the liquid droplet.

Injections into the blastocoelic cavity were performed with micropipettes having outer tip diameters of 0·5 μm, which were firepolished by the puller. The tip was inserted between two lateral micromeres and advanced into the blastocoelic cavity. The injections were performed from immediately before to approx. 20 min after the completion of cytokinesis of 5th cleavage. At these stages, the embryos have a large blastocoelic cavity and the cells are not flattened against each other, which makes it easier to penetrate between cells. The injected amounts varied from 5 to 10 pl. Control injections with 0·05 M-KCl have shown that development remains normal after blastocoelic injections of up to 40 pl.

Processing of embryos

Embryos injected with FD were examined *in vivo* with a fluorescence microscope. Embryos injected with HRP were fixed in either 4% formaldehyde in PBS (0·1 M, pH 7·4) or 2% glutaraldehyde in PBS during at least 15 min. Control experiments have shown that endogenous peroxidase is inactivated during this fixation period. Next, the embryos were washed in PBS and stained for 10 min with a mixture of 1 mg ml^{-1} 3,3′-diaminobenzidin (DAB, Sigma Chemical Company) and 0·01% H_2O_2 in 0·1 M-Tris buffer, pH 7·6, according to Graham & Karnovsky (1966). Proper inactivation of endogenous peroxidase was monitored by means of several uninjected control embryos which were processed in the same way as the injected ones. These control embryos remained completely unstained. After dehydration, clearing in xylene and embedding in Canada Balsam, the embryos were observed in a Zeiss light microscope, either using brightfield or darkfield optics, or Nomarski differential interference contrast for making optical sections.

Electrophoresis

To assess the purity of the HRP preparations that have been used, they were analysed under nondissociating conditions in a discontinuous Tris/glycine sodium dodecyl sulphate electrophoresis system as described by Laemmli (1970) using a 4·5–26·8% gradient running gel and a 4% stacking gel, in a Biorad slab gel apparatus. Stock solutions were prepared according to Lugtenberg *et al.* (1975) and the gradient gel according to Tuszinski, Buck & Warren (1979). Acrylamide was purchased from Serva and recrystallized according to Loenig (1967). Methylene bisacrylamide and a molecular weight standard mixture were purchased from Biorad. 10 or 20 μl samples of HRP (1 mg ml^{-1}), MP II microperoxidase (1 mg ml^{-1}, Sigma Chemical Company) and the molecular weight standard mixture were loaded on

duplicate wells. Electrophoresis was carried out at 25 mA constant current. Different gels were stopped at varying times before or after the ionic front leaves the gel. This procedure ensured that no low molecular weight components were missed, either because they had already left the gel, or because they were not yet completely focused. One part of the gel was stained in Coomassie Brilliant Blue R250 (0·25 % in 50 % methanol and 10 % acetic acid in *aqua bidest*) and the other part was washed twice for 5 min in PBS and then stained in DAB/H_2O_2 for 15 min. Longer periods did not result in a more intense staining, even when staining was performed overnight. The staining reaction was stopped by washing in PBS and the gel was fixed in methanol/acetic acid.

Results

Embryos injected by iontophoresis or by pressure continued to develop normally and in step with control embryos, so we concluded that neither the injection procedure nor the injected substances were harmful. The results achieved with pressure injections and with iontophoresis were essentially the same. This indicates that electrical stimulation of the impaled cell during iontophoresis did not influence the results. Also, there was no visible difference in results or embryonic development, whether the pressure injections were performed with the tracers dissolved in *aqua bidest* or in 0·05 M-KCl. In the following, an embryo at stage x min after the completion of cytokinesis of 5th cleavage will be referred to as an embryo at stage V + x.

Intracellular injections

As we were especially interested in the communication between the mesoderm-inducing animal micromeres and the presumptive mesodermal stem cell (3D), our investigation was focused on the spreading of label between these two cell types. Although all four macromeres of a 32-cell-stage embryo have the capacity to give rise to the mesodermal stem cell (van den Biggelaar & Guerrier, 1979), usually one of the two vegetal cross-furrow macromeres will actually do so, because these have a slightly advantageous spatial position within the embryo as compared to the two other macromeres. If one of these cross-furrow macromeres is injected, there will be a probability of approx. 50 % that this will turn out to become 3D. At later stages (at V + 50–60, i.e. when contact with the overlying animal micromeres is established), 3D has attained a central position in the embryo and can be readily identified. The results described below represent a summary of a total of more than 200 successful impalements.

Single blastomeres of embryos at stages varying from the 2-cell to the early 32-cell stage were injected with tracers (HRP II, HRP VI, FD). Both types of HRP gave essentially the same results, but as the specific activity of HRP type VI is much higher than that of HRP type II, the clearest results were obtained with the former. When injected embryos were investigated before V + 50–60, labelling was restricted either to the impaled cell or to its offspring in cases where one or more cleavages were allowed between injection and tracer visualization (Fig. 1). However, an unmistakable spreading of HRP-label was observed when fixation was performed at V + 50–60 or later.

When HRP was injected into the future 3D macromere, and fixation and staining of the embryo was

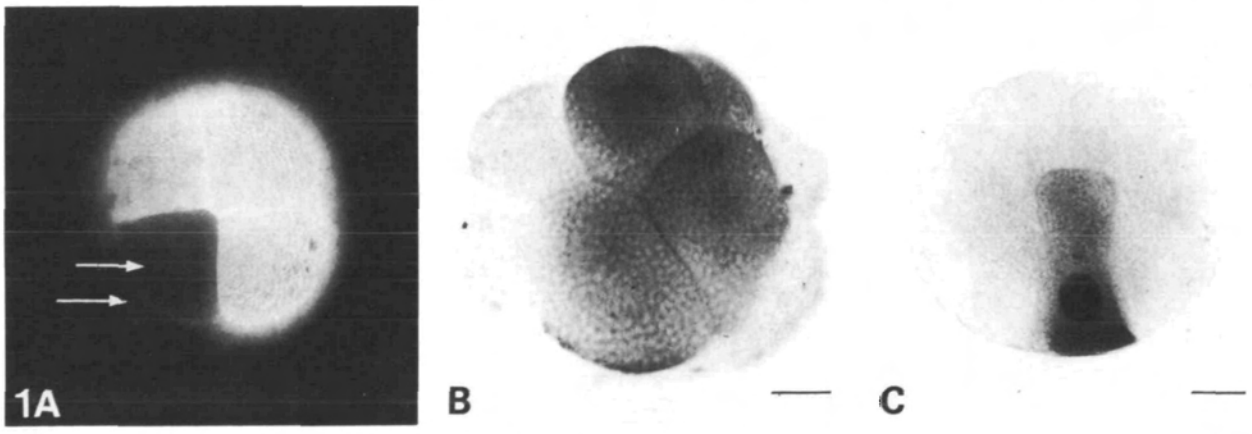

Fig. 1. Intracellular injections of HRP in *P. vulgata* embryos. The embryos were fixed before the transfer of label started. (A) Animal pole view of an embryo injected at 4-cell stage and fixed at 8-cell stage. Only the two daughter cells of the injected cell are labelled (arrows). Darkfield optics. (B). Embryo injected at 4-cell stage and fixed at 16-cell stage. Only the four progeny cells of the injected cell are labelled. Note the spiral cleavage pattern that is typical for equally cleaving molluscs. Brightfield optics. (C) Optical section of an embryo of which the central 3D macromere was injected at V + 30 and fixed at V + 50. The embryo was fixed just before the transfer of HRP would start. Note the strong labelling of the nucleus. Nomarski optics. Bar, 25 μm.

performed at V + 50–60 or later, the overlying animal micromeres were stained, as well as the blastomeres surrounding 3D at the vegetal pole (Fig. 2). The spreading of stain was fast: within a few minutes after an injection, the first nondescendant blastomeres were positively labelled. When an embryo was fixed this soon after injection, apart from the injected 3D cell, only a few cells in the animal pole region were stained. When more time elapsed between injection and fixation, more cells were labelled (Fig. 2C).

The possibility cannot be excluded that the animal micromeres received the label indirectly from the vegetally and meridionally located cells rather than directly from 3D. This is unlikely, however, as distribution of HRP over the blastomeres is preceded by an intermediate phase in which the animal and vegetal cells are more heavily labelled than the equatorial cells interposed between them (Fig. 2A). When the macromeres 3A, 3B or 3C were injected, spreading of label from the impaled cell to surrounding cells was also found, starting at the contact stage (V + 50–60). Spreading was found even when only a very small amount of HRP was injected. Fig. 2B shows such an injection into one of the most animal micromeres. Note that in these cases HRP was first detected in the nuclei (Fig. 2B).

As HRP was detected by means of DAB/H_2O_2 staining after fixation of the injected embryo, it could

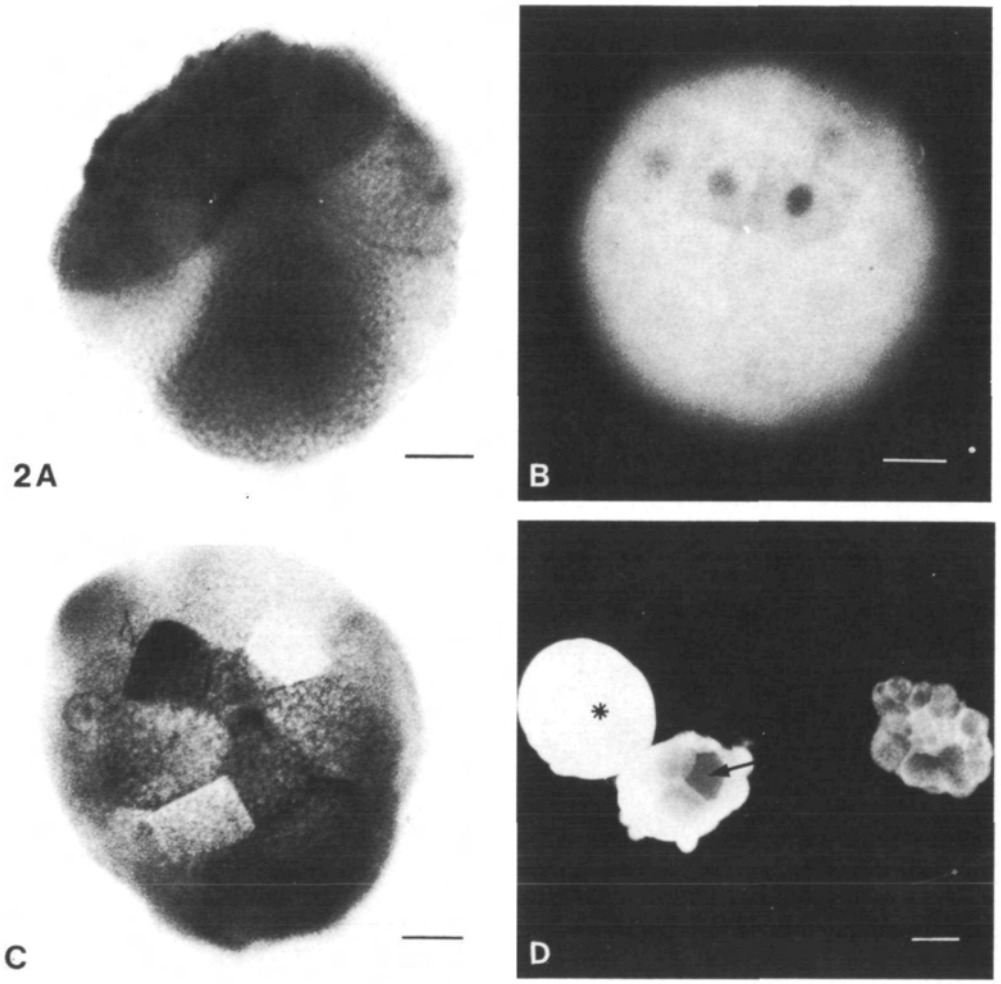

Fig. 2. Intracellular injection of HRP in *Patella* embryos. The embryos were fixed after the transfer of label started. (A) Brightfield optical section of a *P. vulgata* embryo injected with HRP VI at V + 10 in one of the two cross-furrow macromeres. The injected macromore turned out to be the central 3D macromere in this experiment. The embryo was fixed at V + 60. Note that the equatorial cells are less labelled than the injected 3D macromere and the overlying micromeres. (B) Animal pole view of *P. vulgata* embryo injected with HRP II in one of the most animally located micromeres at stage V + 45. The embryo was fixed at V + 50. Only some animal micromeres appear to be labelled. Note the strong labelling of the nuclei. Darkfield optics. (C) *P. coerulea* embryo injected with HRP VI at V + 20 in one of the vegetal cross-furrow macromeres and fixed at V + 75. Most cells are stained. The differential staining pattern as represented by this embryo is more clear in *P. coerulea* than it is in *P. vulgata*. Brightfield optics. (D) *P. vulgata* embryo injected with HRP VI at V + 70 in 3D (arrow) and bisected at V + 75, prior to fixation. Both halves contain HRP-positive cells. The asterisk indicates an uninjected control embryo. Darkfield optics. Bars: A,B,C, 25 μm; D, 50 μm.

not be excluded that HRP might diffuse aspecifically during fixation or that the staining product might diffuse. Therefore, impaled embryos were divided into animal and vegetal halves prior to fixation by use of a glass needle. After such an experiment, cells in both halves appeared to be stained, indicating that it must have been the HRP in its intact form that spread in the living embryo (Fig. 2D).

Blastocoelic cavity injections

Although, at this stage of the investigation, it is not yet clear which mechanism is responsible for the transfer phenomenon described above, it seems likely that the uptake of label in the recipient cells may be a process related to endocytosis. To investigate this possibility, we decided to inject tracers into the blastocoelic cavity, although the mechanism(s) for the uptake of substances from the blastocoelic cavity may not necessarily be identical to the mechanism(s) that cause intercellular transfer.

When HRP was injected into the blastocoelic cavity at late 4th cleavage stages or at early stages after 5th cleavage, and fixation and staining of the embryo were performed before it had reached stage V + 50–60, the label was found only in the blastocoelic cavity and not in the cells, except occasionally in a cell that was damaged, but not killed, during the injection (Fig. 3A). When processing of an injected embryo was performed later than V + 60, many cells were positively labelled, often in a very regular pattern (Fig. 4). Thus, these results were analogous to the results obtained with intracellular injections of HRP as far as the start of label spreading was concerned. After injection in the blastocoelic cavity, however, the results were clearer, probably because the amount of HRP injected into the blastocoelic cavity was higher than the amount injected into a cell. Shortly before the contact stage was reached, the injected HRP accumulated near the membranes facing the blastocoelic cavity. It was not possible to establish from optical sections whether the HRP accumulated inside or outside the cells (Fig. 3B). If it was inside the cells, this might indicate that the first uptake started shortly before the contact between 3D and the micromeres was established.

From the above results, we could not exclude the possibility that the observed spreading of label after injection of HRP into the blastocoelic cavity might be due to the movement of label during or after fixation. Therefore, we injected FD into the cleavage cavity at various stages after 5th cleavage and observed the injected embryos *in vivo*. We never observed uptake of FD from the blastocoelic cavity, not even after the prolonged presence of FD (Fig. 3C). The cells of the injected embryos seemed to show a diffuse labelling, but from experience we know that this type of image is due to internal reflections, in this case caused by the FD in (remainders of) the blastocoelic cavity. When Fig. 5B is compared to Fig. 3C, it is obvious that massive uptake of FD looks different from the result represented in Fig. 3C.

The difference in behaviour of HRP and of FD after blastocoelic cavity injections again raised the question whether the spreading of HRP was due to aspecific diffusion during or after fixation, or whether real intercellular transfer occurred. Therefore, we decided to inject a mixture of HRP and FD (see Fig. 5). Whereas before the contact stage neither HRP nor FD was internalized (Fig. 5A), both were taken up at stages later than approx. V + 60 (Fig. 5B). A slight shift towards yellower wavelengths of the FITC fluorescence was visible after the

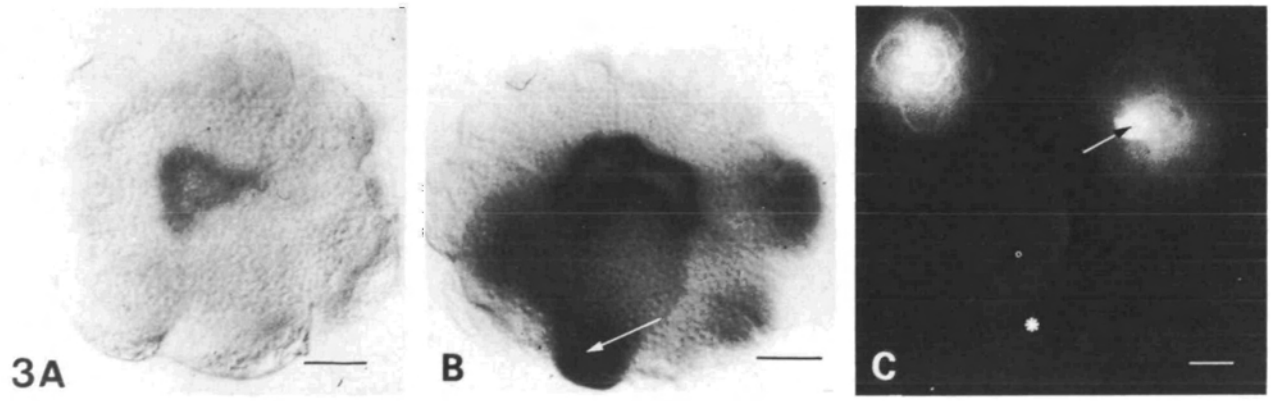

Fig. 3. Blastocoelic cavity injections of HRP and of FD in *P. vulgata* embryos. The embryos were fixed before the start of uptake. (A) Nomarski optical section of an embryo injected with HRP VI at V + 10. The embryo was fixed at V + 40. HRP is only present in the blastocoelic cavity. (B) Nomarski optical section of an embryo injected at V + 10 and fixed at V + 50. The cell indicated by the arrow was probably damaged, but not killed, during the injection. (C) Fluorescence photomicrograph of living embryos injected with FD only at V + 20. The micrograph was taken at V + 95. The asterisk indicates an uninjected control embryo. Due to internal reflections, the cells seem to be positively labelled. The arrow indicates a cell that was damaged during the injection. Bars: A,B, 25 μm; C, 50 μm.

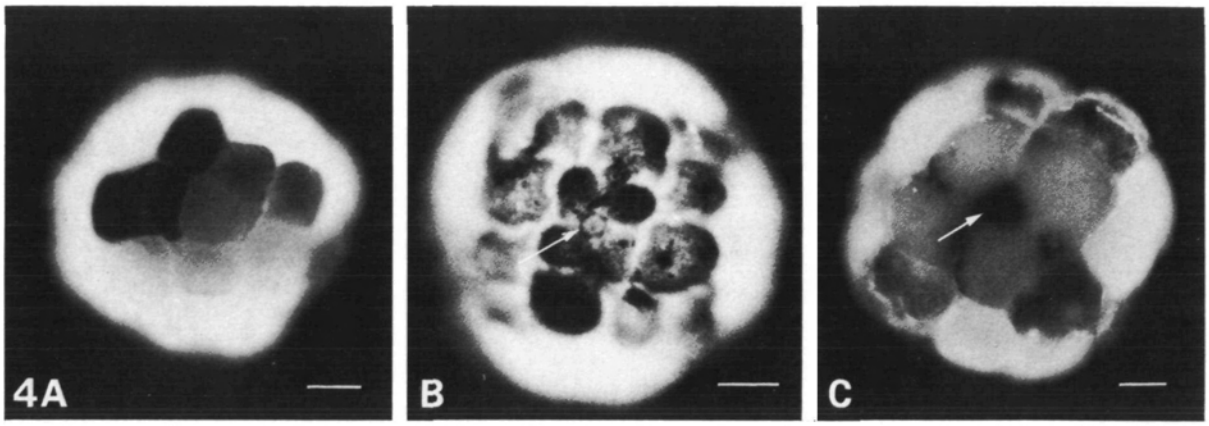

Fig. 4. Blastocoelic cavity injections of HRP VI in 32-cell stage *P. vulgata* embryos. The embryos were fixed after the start of uptake. (A) Embryo injected at V + 5 and fixed at V + 50. The HRP has been taken up by several micromeres. Darkfield optics. (B) Animal pole view of an embryo injected at V + 30 and fixed at V + 60. Only the second quartet micromeres are unlabelled. The arrow indicates the polar bodies. Darkfield optics. (C) Vegetal pole view of the embryo shown in B. Also the four macromeres are positively labelled. This embryo was impaled between the two cross-furrow macromeres. The arrow indicates HRP that leaked out during the injection. Darkfield optics. Bars, 25 μm.

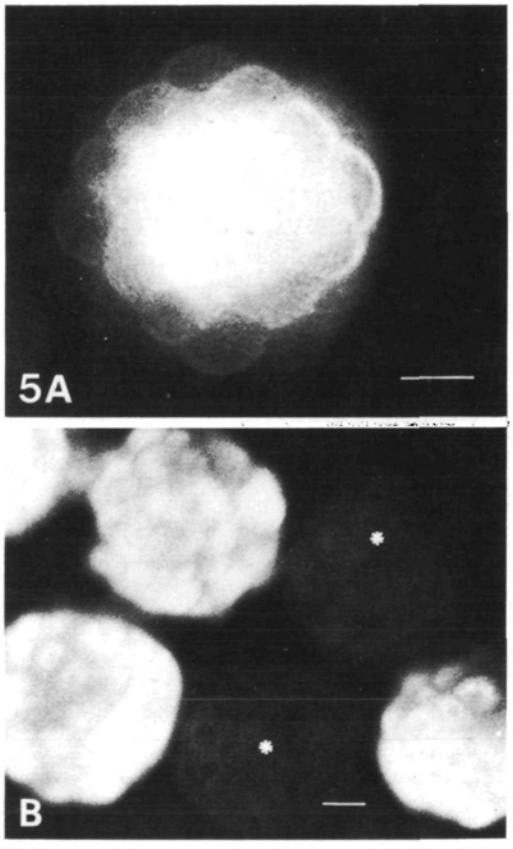

Fig. 5. Fluorescence photomicrographs of living *P. vulgata* embryos injected in the blastocoelic cavity with a mixture of HRP VI and FD. (A) Embryo injected at V + 10 and photographed at V + 30. A large blastocoelic cavity is still present. The cells are still unlabelled at this stage. (B) Embryos injected at V + 20 and photographed at V + 75. The FD is now taken up by the cells. Uninjected control embryos are indicated by asterisks. Bars, 25 μm.

FD was internalized by the cells. This was not quantified, but only assessed by visual examination. The whole embryo was FD labelled, including the cells that remained unstained when tested for the presence of HRP (cf. Fig. 4). In contrast, no uptake of FD in any cell was observed when it was injected alone. Another difference between the behaviour of FD and HRP was that at very late 5th cleavage stages (i.e. V + 90–100 or later), the fluorescence of FD was still present, whereas no HRP was detectable any more, except for HRP inside a cell that was damaged during the injection.

Electrophoresis

Before one can conclude that the 32-cell embryo of *Patella* must have an intercellular transfer mechanism for HRP, one has to be sure that the HRP preparations do not contain stainable degradation products small enough to pass through gap junctions. DAB/H_2O_2 and Coomassie Brilliant Blue staining of HRP preparations on SDS–polyacrylamide gels did not reveal low molecular weight components, even though the amount of HRP loaded on a well was in the order of 5×10^6-fold in excess of the estimated amount injected into a cell (Fig. 6).

Discussion

The results described in this paper clearly show that HRP is not a reliable cell lineage tracer in each developmental system. In *Patella*, HRP is transferred from a well-defined stage onwards. The time at which the observed phenomenon starts is precisely determined at V + 50–60. Such a consistent timing in an

embryonal system argues in favour of a physiologically and developmentally significant communication event. The start of tracer spreading coincides with, or only slightly precedes, the contact between the central macromere 3D and the overlying micromeres. Therefore, the possibility should be considered that the observed spreading reflects a mechanism that might play an important role in the interaction between 3D and the micromeres, leading to the induction of the mesodermal cell line.

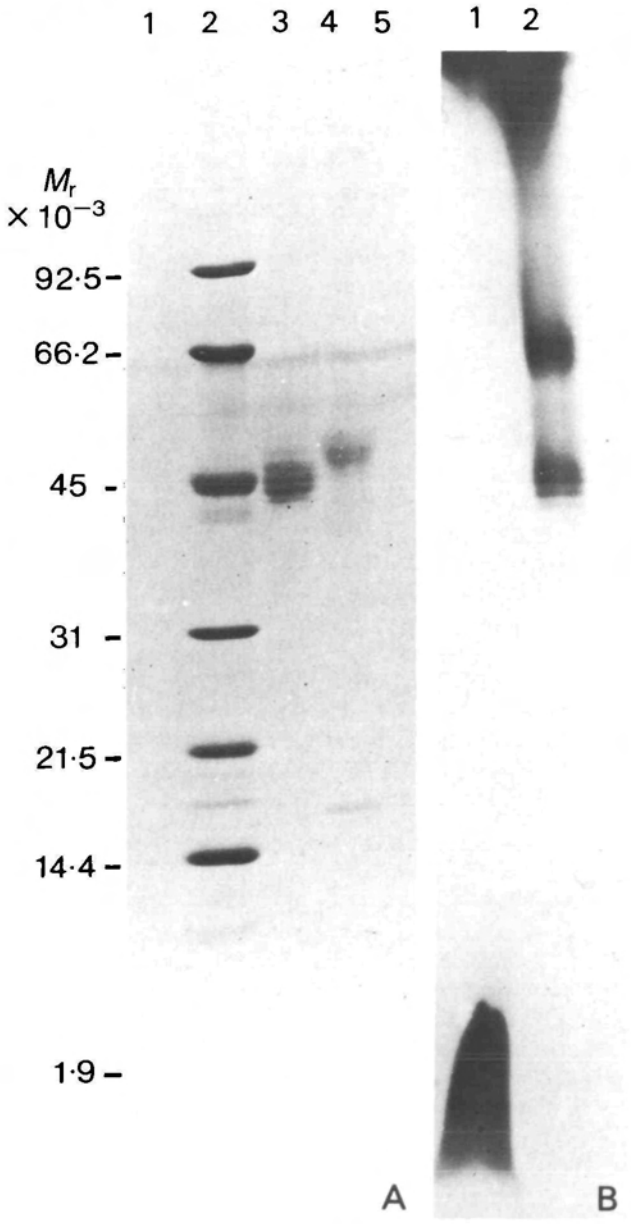

Fig. 6. Gradient polyacrylamide gel electrophoresis of HRP samples did not reveal low molecular weight components small enough to pass through gap junctions. (A) Coomassie Brilliant Blue staining. **1**, microperoxidase MPII ($10\,\mu g\,10\,\mu l^{-1}$); **2**, Biorad low molecular weight standard mixture (lysozyme, soy bean trypsin inhibitor, carbonic anhydrase, ovalbumin, bovine serum albumin and phosphorylase B); **3**, HRP type II ($10\,\mu g\,10\,\mu l^{-1}$); **4**, HRP type VI ($10\,\mu g\,10\,\mu l^{-1}$); **5**, microperoxidase MPII ($20\,\mu g\,10\,\mu l^{-1}$). Note that the microperoxidase is not stained. (B) DAB/H_2O_2 staining. **1**, microperoxidase ($20\,\mu g\,10\,\mu l^{-1}$); **2**, HRP type VI ($20\,\mu g\,20\,\mu l^{-1}$).

Before discussing the possible nature of the transfer mechanism(s) involved, we will first evaluate the question whether the observed spreading might be due to an artefact. First, the strongest argument against artefact comes from the experiments in which a HRP-injected embryo is bisected before fixation. The results of these experiments indicate that intercellular transfer of HRP has occurred in the living embryo. Second, the moment at which transfer of HRP can first be observed is reproducible and phase specific. Third, it has been found that aspecific spreading of HRP can result from overloading axons in axophoresis experiments (Brown & Fyffe, 1984). However, this cannot be the case in our experiments, as spreading of label is also observed when only a small amount of HRP is injected (Fig. 2B). Moreover, it would be difficult to explain why overloading in *Patella* should occur at V + 50 or later and not before that stage, and why the nuclei are labelled first. Fourth, Bennett (1973) has shown in *Fundulus* that nonspecific spreading of HRP after fixation will occur when fixation is performed in the presence of $La(OH)_3$, but not when normal fixation procedures are used.

The question then arises whether the transfer of native HRP molecules accounts for the intercellular spreading of label or whether this is the result of transfer of low molecular weight degradation products through gap junctions. From the absence of low molecular weight subunits of HRP on SDS–polyacrylamide gels, we conclude that HRP is transferred in its native, high molecular weight form, although it cannot be excluded that HRP is degraded intracellularly, perhaps into components small enough to pass gap junctions. The latter assumption, however, is very unlikely, as it would be difficult to explain why this would not happen at earlier stages. Dorresteijn *et al.* (1983) have shown that spreading of Lucifer Yellow in *Patella* starts at V + 10–20. Moreover, embryos that have been injected intracellularly with HRP at stages V + 50 or later, and fixed immediately after completion of the injection, already show a considerable amount of spreading of DAB/H_2O_2-positive material. Extensive intracellular degradation of HRP in such a short time is highly unlikely. Therefore, we may conclude that it is the intact HRP molecule that is transferred intercellularly.

Three generally accepted mechanisms for the intercellular spreading of HRP can be envisaged: (1) passage through midbodies, as suggested by Goodall & Johnson (1984); (2) passage through large intercellular channels, e.g. in the form of cytoplasmic

bridges as found in the squid embryo (Cartwright & Arnold, 1980); Ginzberg, Morales, Spray & Bennett, 1985) and in the zebrafish embryo (Kimmel & Law, 1985) or (3) exocytosis followed by endocytosis as found in synapses (Hongo *et al.* 1981; Triller & Korn, 1981).

Transfer *via* midbodies is very unlikely, as there is no reason why they would not permit transfer before stage V + 50. The presence of intercellular channels large enough for HRP to pass through cannot be excluded, although in previous electron microscopical studies no indications have been found for cytoplasmic bridges (Dorresteijn *et al.* 1982). The third possibility, exocytosis of HRP followed by endocytosis by adjacent cells seems to be a likely one. If endocytosis occurs, it is to be expected that the HRP/FD mixture will be taken up into lysosomes. The low pH of the lysosomal fluid would account for the observed shift in fluorescence of FD towards longer wavelengths (Martin & Lindqvist, 1975; Geisow, 1984). Both FD and HRP would be digested in the lysosome, rendering the HRP inactive and liberating FITC, which is small enough to pass through gap junctions. The free FITC could therefore spread throughout the whole embryo. This is in accordance with the observation that at later stages the DAB/H_2O_2 reaction for HRP is negative, whereas fluorescence by FITC is still present.

Another factor to be considered is that HRP and FD are taken up by the micromeres, in spite of the fact that these cells are covered with a layer of extracellular matrix shortly before and during the contact phase (Kühtreiber *et al.* 1986). HRP has been shown to be able to pass through such a layer. Modespacher, Rudin, Jenni & Hecker (1986) have shown that HRP can pass the basal lamina of the midgut of the tsetse fly and is taken up into fatbody cells. Straus (1981, 1983*a*,*b*) has shown that several cell types of the rat have mannose-specific binding sites for HRP. The binding of HRP to these receptors can be suppressed by competition with mannose or glycoproteins. As the ECM of *Patella* micromeres reacts positively with mannose-specific lectins and contains glycoproteins (Kühtreiber *et al.* 1986), similar binding sites may be present in a 32-cell-stage *Patella* embryo. This implies that these embryos may possess an uptake mechanism for ECM, that enables HRP to utilize a receptor-mediated endocytosis mechanism to enter the cells by binding to the receptors normally utilized by ECM components.

Our results suggest that HRP stimulates the endocytosis process that would normally occur at a lower level in uninjected or FD-injected embryos. In the presence of HRP the stimulation of endocytosis would facilitate the nonspecific entry of FD into the cells, whereas in the absence of HRP the rate of uptake of FD would be much lower and thus not detectable. This might also explain why Dorresteijn *et al.* (1983) could not detect the direct transfer of Lucifer Yellow between the central 3D macromere and the contacting micromeres. The mechanism proposed above can explain how HRP gets into the cells. However, it does not explain how it gets out of them. To explain the rate of endocytosis, one has to assume that exocytosis is also stimulated. This remains to be explained.

Summarizing, we may conclude first, that embryos of *Patella vulgata* and *P. coerulea* have a mechanism for the transfer of specific substances at the 32-cell stage. This mechanism may be stimulated by HRP and must be different from junctional channels. Further experiments are in progress to elucidate the nature of this mechanism. Second, at least in these molluscan embryos, HRP cannot be used as a reliable marker in cell lineage studies.

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