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# Inductive regulation of cell fusion in leech

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## SUMMARY

**Cell-cell fusion is a component of many different developmental processes, but little is known about how cell-cell fusion is regulated. Here we investigate the regulation of a stereotyped cell-cell fusion event that occurs among the endodermal precursor cells of the glossiphoniid leech *Helobdella robusta*. We find that this fusion event is regulated inductively by a cell that does not itself fuse. We also show that biochemical arrest (by microinjection with ricin A chain or ribonuclease A) of the inducer or either of the fusion partners prevents fusion, but only if the arrest is initiated during a critical period long before the time at which fusion normally occurs. If the arrest occurs after this critical period, fusion occurs on schedule. These results suggest that both fusion partners play active roles in the process and that neither the induction nor the fusion itself requires concomitant protein synthesis.**

Key words: Annelid, Leech, *Helobdella robusta*, Cell fusion, Endoderm, Ricin A chain, Syncytium

## INTRODUCTION

Syncytial yolk cells (SYCs) are a prominent embryonic feature in many annelid and arthropod species (Anderson, 1973). The best known example is the syncytial blastoderm of *Drosophila melanogaster*, which arises directly from the zygote by nuclear proliferation. Upon cellularization, the *Drosophila* blastoderm contains the precursor cells for all the different parts of the embryo. In various wingless insects, such as silverfish and springtails, the SYC gives rise to midgut epithelium either exclusively or by a second wave of cellularization (after that which forms ectoderm and mesoderm; Anderson, 1973). There is also variability in the processes by which SYCs form; in springtails, SYC formation involves cell fusion, rather than exclusively nuclear proliferation without cytokinesis.

Similar fusion processes operate in annelid embryos. Specifically, in the glossiphoniid leech *Helobdella robusta*, the SYC which eventually cellularizes to form the lining of the mature gut (Nardelli-Haefliger and Shankland, 1993) arises by a series of stepwise and stereotypic cell-cell fusions (Liu et al., 1998; Fig. 1. Early in gastrulation, between 51 and 63 hours after zygote deposition (AZD), two large, yolky, endodermal precursors (cells A‴ and B‴) fuse with one another to form cell A/B. Near the end of gastrulation, approximately 25 hours later, a third large yolk cell (cell C‴) fuses with A/B to form A/B/C. Later still, the bilaterally paired teloblasts (progenitors of the mesodermal and ectodermal segmental tissue and descendants of blastomere D of the 4-cell embryo) cease their stem cell-like divisions and fuse with A/B/C to form the SYC (Schmidt 1939; Liu et al., 1998). The segmental founder cells of the teloblasts that are not incorporated into the definitive segmental plate also fuse with this syncytium (Desjeux and Price, 1999; Shankland, 1999). Thus, the SYC is composed of nuclei not only from the three endodermal lineages (descendants of blastomeres A, B, and C of the 4-cell embryo) but also from the mesodermal and ectodermal lineages (descendants of blastomere D of the 4-cell embryo).

Cell-cell fusion is a component of many different developmental processes. Sperm and egg fuse during fertilization (Yanagimachi, 1988); yeast fuse during mating (McCaffrey et al., 1987); myoblasts fuse during muscle development (Knudsen, 1991); cytotrophoblasts fuse during placental development (Pierce and Midgley, 1963); monocytes fuse to form osteoclasts involved in bone resorption (Baron et al., 1993); primary mesenchyme cells fuse during formation of the sea urchin larval skeleton (Hodor and Ettensohn, 1998); and cell fusions occur in the formation of epithelia in the nematode *C. elegans* (Podbilewicz and White, 1994; Sharma-Kishore et al., 1999).

Despite the ubiquity of cell fusion in development, little is known about how the process is regulated (Hernandez et al., 1996). In *C. elegans*, it has been shown that cells are induced to fuse to a pre-existing syncytium by signals emanating from the syncytium itself (Herman and Hedgecock, 1990), and that separate parts of a fusion-competent cell can fuse to one another in the absence of the normal fusion partner (Sharma-Kishore et al., 1999). Here, we show that fusion can also be regulated nonautonomously, by cells that do not themselves undergo fusion.

## MATERIALS AND METHODS

### Embryos

*Helobdella robusta* embryos were obtained from a laboratory breeding colony (Shankland et al., 1992) and cultured at 23°C in daily changes of HL saline (4.8 mM NaCl, 1.2 mM KCl, 2.0 mM $MgCl_2$, 8.0 mM $CaCl_2$, and 1.0 mM maleic acid, pH 6.6) supplemented with antibiotics [0.2 mg/ml gentamicin (Sigma); 50 Units/ml penicillin, 50 μg/ml streptomycin (Gibco)], unless otherwise indicated. The developmental staging system and cell nomenclature is modified from that of Stent et al. (1992). Fused cells are designated as per Liu et al. (1998; Fig. 1). Macromeres, proteloblasts and teloblasts are designated by capital letters. Micromeres are designated by lowercase letters corresponding to the name of their parent blastomere. Primes (′) are used to designate the birth order of the resulting micromeres and blastomeres. When referring to a specific macromere or proteloblast lineage irrespective of how many micromeres it has made, a superscript 'x' is added.

### Fusion assay

The fusion of the A and B lineage macromeres was assayed by the diffusion of lysinated, fluorescent dye-conjugated dextrans or β-galactosidase from $A^x$ into $B^x$ and/or vice versa. Due to their large size (10 kDa and 130 kDa, respectively) these lineage tracers were confined to the injected cell and its mitotic progeny unless cell fusion occurred between a labeled and unlabeled cell, in which case the lineage tracer diffused into the fusion partner. To assay for cell fusion using fluorescence, $A^x$ and $B^x$ were each micro-injected during stages 3-6a with a different fluorescent lineage tracer [rhodamine-conjugated dextran amine (RDA) or fluorescein-conjugated dextran amine (FDA)] as described by Nardelli-Haefliger and Shankland (1993). At selected times, embryos were fixed overnight at 4°C or for 1 hour at ambient temperature (4% formaldehyde in 100 mM Tris-HCl, pH 8.0), rinsed (several changes of 100 mM Tris-HCl), cleared with 100% glycerol, and viewed in whole mount with epifluorescence microscopy (Zeiss Axiophot). Fusion between $A^x$ and $B^x$ was judged to have occurred if RDA and FDA were coincident within the embryo. On the other hand, if the RDA and FDA were in separate and distinct regions then fusion had not occurred. In some instances, embryos were assayed for fusion live, before fixation and clearing.

To assay for cell fusion using histochemical detection of β-galactosidase, a solution containing β-galactosidase was micro-injected into either $A^x$ or $B^x$ sometime during stages 3-6a. Injection, fixation, histochemical detection of the β-galactosidase, and the scoring of fusion were as previously described (Liu et al., 1998).

### Embryo dissections

To determine if A‴-B‴ fusion occurred despite the removal of a specific cell from the living embryo, the vitelline envelope of previously injected embryos (as described above) was first removed. Devitellinized embryos were prepared and cultured on a sterile bed of 0.35% agarose in HL saline. The vitelline envelope was ruptured by bathing the stage 4a embryos for 2-15 minutes in a solution of 10 mM dithiothreitol (DTT) and 0.1% trypsin (Sigma) in HL saline with 16 mM $CaCl_2$, pH 8.1 (HLC saline); DTT was added to previously frozen aliquots of trypsin in HLC. Immediately after vitelline envelope rupture, embryos were rinsed twice by transfer with a fire-polished glass pasteur pipet to a fresh dish of HLC saline; the vitelline envelopes came off in the course of these transfers.

Devitellinized embryos were then moved to a fresh Petri dish containing HL saline lacking $Mg^{2+}$ and $Ca^{2+}$ ions (zero divalent HL saline; 19.8 mM NaCl and 1.2 mM KCl). The cell of choice was then dissected from the embryo with a fire-polished tungsten needle (California Fine Wire Company, size 0.002) or a cat-hair knife (Keller, 1991). Dissection tools were rinsed with 95% ethanol for sterilization purposes, and the ethanol was allowed to evaporate for >2 hours. Embryos in which more than approximately 5% of the dissected cell remained attached or in which a neighboring cell was damaged during the procedure were discarded. After dissection, embryos were moved to a fresh dish of HLC saline supplemented with antibiotics which was changed daily. Similarly operated sibling embryos were cultured together.

For each clutch of embryos prepared for dissection, several embryos were devitellinized but left undissected to serve as controls. The entire clutch was discarded if the control embryos did not survive or if less than 40% of them exhibited A‴-B‴ fusion. Individual embryos which turned white, lost their smooth contours, divided abnormally, or developed large vacuoles were discarded.

A‴-B‴ fusion was scored using epifluorescence microscopy when the embryos were at least 77 hours AZD. This corresponds to at least 66 hours after the dissections were performed and ≥15 hours after A‴-B‴ fusion occurs in 100% of unmanipulated embryos (Liu et al., 1998).

### Biochemical arrest of individual cells

To biochemically arrest individual cells, the cell of choice was micro-injected with either 45 μg/ml ricin A chain from *Ricinus communis* (Sigma) or 0.5 mg/ml RNase A; each in 1% fast green and 0.05 N KCl. In addition, FDA and RDA or ß-galactosidase was injected into the A and/or the B lineage macromere so that fusion could be monitored (see fusion assay above). Embryos were cultured until 68-83 hours AZD and then fixed and processed as above. The time of fixation corresponded to 5-20 hours after fusion occurs in 100% of control embryos (Liu et al., 1998).

### Photography and imaging

Live, operated embryos and fixed, β-galactosidase-injected embryos were photographed using Ektachrome 160 film (Kodak) with dark-field or DIC optics, respectively (Zeiss Axiophot). Images of RDA- and FDA-injected embryos were photographed with Ektachrome 400 film or are a z-series projection of roughly 10, 5-μm optical sections taken with a scanning confocal microscope (BioRad MRC600). Images were processed using Adobe Photoshop 4.0 and Illustrator 6.0.

## RESULTS

### Summary of leech development

Development of glossiphoniid leeches such as *Helobdella robusta* is characterized by stereotyped cell divisions yielding individually identifiable cells with predictable fates. Figure 1 summarizes aspects of *Helobdella* development relevant to the present study. Second cleavage gives rise to blastomeres A, B, C and D (stage 3) which approximate quadrants of a sphere. Progeny of each of these blastomeres are referred to as being from the A, B, C and D quadrants or lineages. The A, B and C lineages are the main progenitors of the endoderm while the D lineage gives rise primarily to the segmental ectoderm and mesoderm (Nardelli-Haefliger and Shankland, 1993; Liu et al., 1998; Weisblat et al., 1984).

Cells A, B and C each divide unequally three times to generate a total of three large, yolky macromeres and nine micromeres (Fig.1, stage 4). The a′, b′ and c′ micromeres result from the first round of division after which the three macromeres are referred to as A′, B′ and C′. Each successive division is denoted by another prime (′) so that the third and final division results in macromeres A‴, B‴ and C‴ and micromeres a‴, b‴ and c‴. Micromeres lie near the animal pole and contribute to the squamous epithelium and to definitive unsegmented prostomial tissues (Weisblat et al.,

1984; Nardelli-Haefliger and Shankland, 1993; Smith and Weisblat, 1994).

The divisions within the D lineage are quite different. After making the d′ micromere, D′ divides to form the mesodermal progenitor DM and the ectodermal progenitor DNOPQ. These in turn divide to form 16 additional micromeres and 10 bilaterally paired stem cells (teloblasts) which are progenitors of the segmental tissues. Each teloblast undergoes repeated unequal divisions to produce a column (bandlet) of segmental founder cells (blast cells). The five bandlets on each side of the

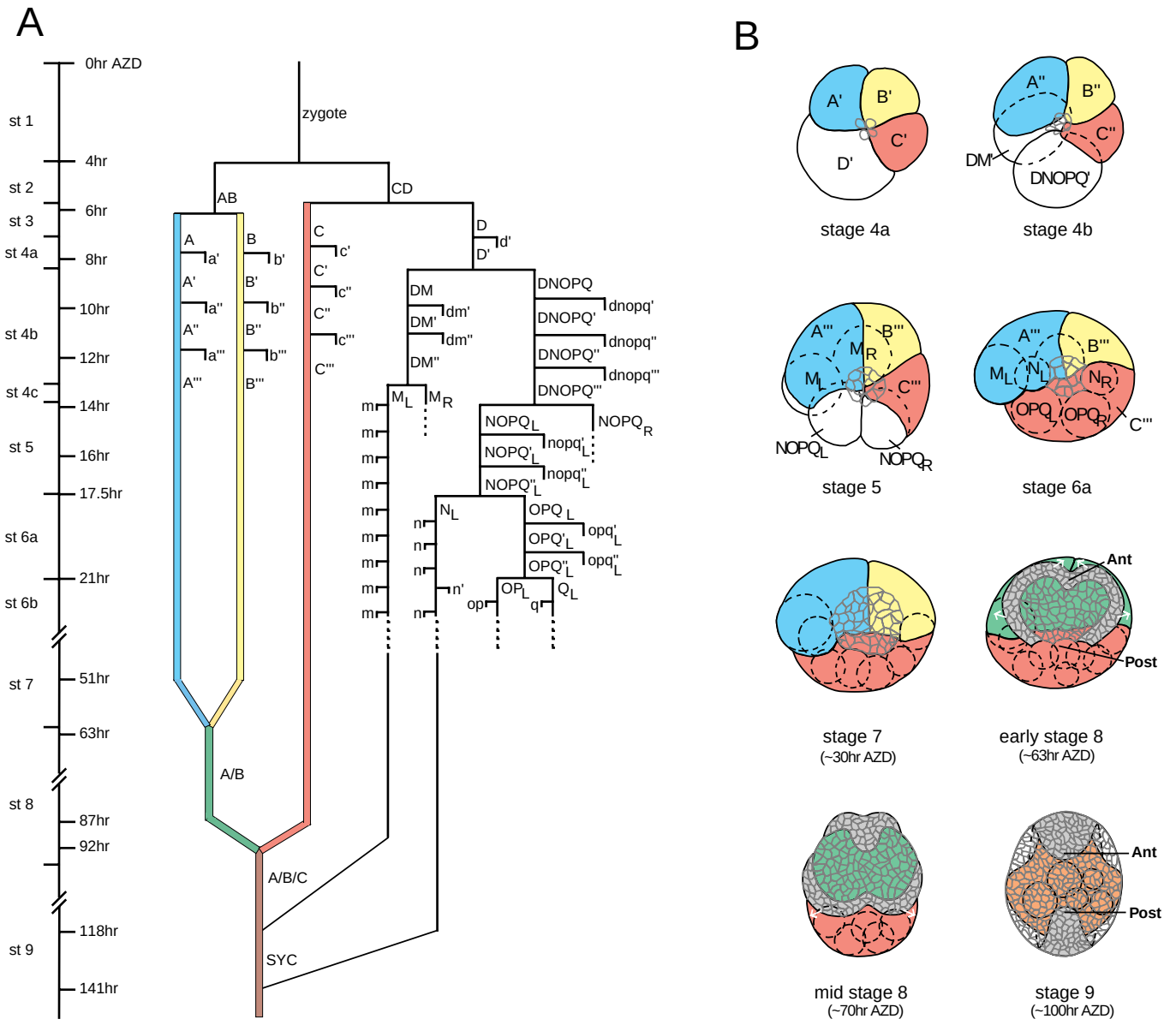



**Fig. 1.** Summary of leech development. The A, B and C lineage macromeres are shaded blue, yellow and red, respectively. A‴ and B‴ fuse to form A/B (green) followed by the fusion of A/B with C‴ to form A/B/C (brown). (A) Cell lineage diagram showing the early cell divisions and stepwise cell fusions that occur to form the endodermal syncytial yolk cell (SYC). The corresponding developmental stages (Stent et al., 1992) and hours after zygote deposition (AZD) are indicated. The names of macromeres, proteloblasts and teloblasts are indicated with capital letters and micromeres and blast cells with lowercase letters (branches to the right and left, respectively). Subscript 'L' and 'R' indicate left and right bilaterally paired cells and lineages. Because the timing of each cell fusion event varies slightly from embryo to embryo, the time window in which each fusion occurs is indicated by diagonal lines connecting fusion partners (Liu et al., 1998). (B) Illustrations of selected stages of embryonic development as seen from the animal pole (prospective dorsal views). During stages 4b-7, the A, B, and C quadrant macromeres spread, relative to the D quadrant, and come to envelop the D quadrant teloblasts. Micromeres, the small, unlabeled cells born to the animal pole region, spread over the embryo surface during stage 6b-9. By stage 7, the 10 bilaterally paired teloblasts have formed and are producing blast cells which coalesce to form the left and right germinal bands (grey). At the end of stage 7, A‴ and B‴ fuse to form A/B, followed by the beginning of stage 8 when the germinal bands meet at the prospective anterior (Ant) and gradually coalesce (in the direction of the arrows) from anterior to posterior (Post) along the ventral midline to form the germinal plate. By stage 9, germinal plate formation is complete, and C‴ has fused with A/B to form A/B/C.

embryo come together in parallel to form the left and right germinal bands. During gastrulation, the germinal bands migrate over the surface of the embryo and coalesce along the prospective ventral midline to form the germinal plate from which the segmented mesoderm and ectoderm arise (Fig. 1B).

## Induction of cell-cell fusion

A basic question regarding the regulation of A‴-B‴ fusion is whether the closely timed fusion of these two cells is regulated inductively. (Here we define induction operationally, as the process by which cells are switched from one pathway to another by the influence of an adjacent cell or cells, without reference to any specific biochemical mechanism.) Alternatively, fusion could be regulated autonomously within the A and/or B lineages. To address this question, we dissected away either the D or C quadrant macromere (D′ or C′) from devitellinized stage 4a embryos and cultured the operated embryos until at least 15 hours after A‴-B‴ fusion would normally have occurred. Prior to the dissections, A or A′ was injected with RDA lineage tracer while B or B′ was injected with FDA lineage tracer (or vice versa) so that A‴-B‴ fusion could be assayed (see Materials and Methods).

The results of these experiments reveal that removal of the D quadrant blocks A‴-B‴ fusion while removal of the C quadrant does not (Table 1; Fig. 2). When D′ was removed, fusion never occurred. Yet, these embryos rounded up to fill in the space previously occupied by D′, continued to make micromeres, and maintained an overall healthy appearance (Fig. 2). When C′ was removed, fusion occurred 58% of the time (Table 1). We attribute the reduced incidence of fusion in these experiments to the trauma of the manipulations since even the devitellinized but unoperated controls exhibited only 81% fusion (Table 1). Thus, A‴-B‴ fusion appears to be regulated inductively by the D quadrant and/or its derivatives and does not require the C quadrant. We also note that teloblast and blast cell formation by the D quadrant progeny could proceed independently of the C quadrant and that embryos lacking C′ generated coalescing left and right bandlets and the beginnings of a germinal plate by the time of fixation (approx. 66-77 hours post-dissection; 74-85 hours AZD; Fig. 2).

Next we asked if a part of the D lineage, specifically the mesodermal DM lineage, is sufficient to induce fusion. For this purpose, we dissected away DNOPQ (DM and DNOPQ are the animal and vegetal daughters of D′ at the fourth cleavage) and assayed for A‴-B‴ fusion as before. In embryos from which DNOPQ was removed, DM divided normally, yielding left and right M teloblasts which each produced a column of blast cells. A‴-B‴ fusion occurred 49% of the time in such embryos (Table 1; Fig. 2). We attribute the variable incidence of fusion in these experiments to the trauma of the experimental procedure and variability in the health of the clutches used since similar variations were seen in sibling embryos that were devitellinized but undissected and from which C′ was dissected (Table 1).

## Biochemical arrest of the inducer

The results of the dissection experiments described above support the conclusion that A‴-B‴ fusion is regulated inductively by the D quadrant. But, this experimental technique is limited because (1) the trauma of the procedure makes it difficult to discern or interpret quantitative differences in the results, (2) as the embryos continue to divide the cells of interest become progressively smaller and migrate inward thereby becoming less accessible to dissection, and (3) cell removal experiments do not address what parts, pathways or molecules within the cell are required.

To circumvent these problems, we sought to block the inductive actions of the D quadrant derivatives by a ‘biochemical arrest’ technique. For this purpose, cell(s) of interest were injected with ricin A chain or RNase A. Ricin acts by catalytically inactivating the 28S ribosomal RNA of the 60S ribosomal subunit (Endo and Tsurugi, 1988), and RNase acts by degrading single-stranded RNA. Although we have not directly tested the effect of these substances on protein synthesis in the *Helobdella* embryo, ricin is a potent inhibitor of protein synthesis in all eukaryotes tested including yeast, fly, mouse and man (Olsnes and Pihl, 1982), and RNase should have a similar effect by degrading cytoplasmic RNA. Both ricin (30.6 kDa) and RNase (13.7 kDa) are too large to diffuse through cell membranes and therefore act only in the injected

**Table 1. A‴-B‴ fusion requires the D lineage but not the C lineage; fusion occurs in the absence of DNOPQ$^{x}$**

| | Devitellinized only | | Devitellinized and C′ removed* | | Devitellinized and D′ removed* | | Devitellinized and DNOPQ$^{x}$ removed* | |
|---|---|---|---|---|---|---|---|---|
| *n* clutches‡ | % fusion | *n* | % fusion | *n* | % fusion | *n* | % fusion | *n* |
| 2 | 77% | 20/26 | 50% | 11/22 | – | – | – | – |
| 1 | 92% | 12/13 | 88% | 14/16§ | – | – | – | – |
| 2 | 85% | 11/13 | 82% | 9/11 | 0% | 0/11 | – | – |
| 1 | 89% | 8/9 | – | – | 0% | 0/7 | – | – |
| 1 | 71% | 5/7 | 14% | 1/7 | 0% | 0/7 | 24% | 4/17¶ |
| 2 | 62% | 8/13 | 36% | 5/14 | – | – | 33% | 4/12¶ |
| 1 | 100% | 10/10 | – | – | – | – | 85% | 11/13** |
| 1 | 67% | 2/3 | 67% | 2/3 | – | – | 71% | 5/7‡‡ |
| **Total** | **81%** | **76/94** | **58%** | **42/73** | **0%** | **0/25** | **49%** | **24/49** |

*The percentage and ratio (*n*) of embryos with A‴-B‴ fusion, as scored using the fluorescence method at embryonic age 6a+59 to 61 hours (66-69 hours post-dissection, ≥14 hours after fusion occurs in 100% of control embryos).

‡Each row represents the data from either one or two clutches worth of embryos split into the experimental groups as indicated.

§C″ was removed rather than C′.

¶DNOPQ was removed.

**DNOPQ′ was removed.

‡‡DNOPQ″ was removed.

cell(s). Cells of the leech embryo injected with either ricin or RNase round up and at most undergo one more cell division while the uninjected cells of the embryo develop normally, except for any developmental processes dependent upon the activities of the injected cell (Nelson and Weisblat, 1992; Smith et al., 1996; Liu et al., 1998). Thus, we describe cells injected with ricin or RNase as being biochemically arrested.

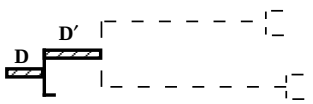
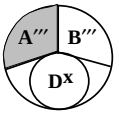
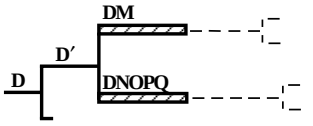
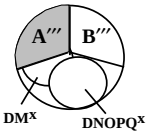
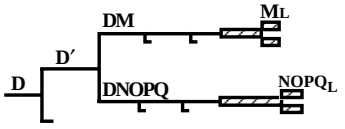
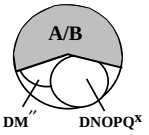
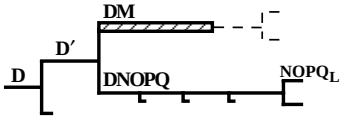
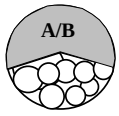
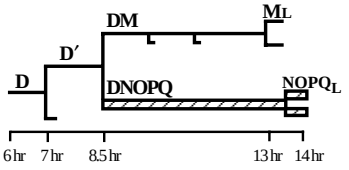
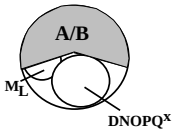
Ricin and RNase injections into selected cells of the embryo confirmed and extended the results obtained from the dissection experiments. When D or D′ was biochemically arrested by ricin injection and the embryo was cultured until at least 11 hours after the time at which fusion normally occurs, A‴-B‴ fusion never occurred (Table 2; Fig. 3A). Similar results were obtained when RNase was used (data not shown).

Conversely, when C, C′, or C″ was injected with ricin, A‴-B‴ fusion occurred in 91% of the embryos (68 out of 75 embryos in 6 experiments). Not only did A‴-B‴ fusion occur, but it occurred during the correct time window; A‴-B‴ fusion did not occur prior to 49 hours AZD but had occurred by 64 hours AZD (data not shown). These results are consistent with those of the dissection experiments and extend those findings by showing that A‴-B‴ fusion is blocked if the D lineage is kept within the embryo but biochemically arrested (i.e. prevented from further divisions and presumably from further protein synthesis).

### Early biochemical arrest of the inducer prevents fusion but late arrest does not

Knowing that the arrest of D′ blocks the fusion of A‴ and B‴, we sought to determine the time course of the inductive influence from the D quadrant. When $DM^x$ and $DNOPQ^x$ (the two daughters of D′) were arrested during the first 2.5 hours after DM and DNOPQ were born (arrest of DM and DNOPQ, DM′ and DNOPQ′, or DM′ and DNOPQ″), A‴-B‴ fusion was largely blocked (Table 2; Fig. 3B). Conversely, when ricin was injected into both $DM^x$ and $DNOPQ^x$ at progressively later times [arrest of DM″ and DNOPQ″; DM″ and DNOPQ‴; or $M_L$, $M_R$ (the two teloblast descendants of DM″) and

**Table 2. Biochemical arrest of the D lineage affects A‴-B‴ fusion**

| Timing and target of ricin injection † | | % embryos with A‴-B‴ fusion ‡ | Schematic result § |
|---|---|---|---|
|  | D<br>D′<br>**$D^x$** | 0<br>0*<br>**0** (0/57) |  |
|  | DM + DNOPQ<br>DM′ + DNOPQ′<br>DM′ + DNOPQ″<br>**early $DM^x$ + $DNOPQ^x$** | 33<br>12*<br>16*<br>**15** (17/112) |  |
|  | DM″ + DNOPQ″<br>DM″ + DNOPQ‴<br>$M_L$ + $M_R$ + DNOPQ‴<br>**late $DM^x$ + $DNOPQ^x$** | 67*<br>69*<br>65*<br>**67** (145/218) |  |
|  | DM<br>DM′<br>DM″<br>**$DM^x$** | 71*<br>85*<br>91*<br>**83** (65/78) |  |
|  | DNOPQ<br>DNOPQ′<br>DNOPQ‴<br>$NOPQ_L$ + $_R$<br>**$DNOPQ^x$** | 76<br>91<br>80<br>86<br>**83** (85/102) |  |

†Partial lineage tree diagrams are of the D lineage (6-14 hours AZD). Cross-hatching indicates the time of ricin injection and the cell that was injected. Dashed lines indicate cell divisions that did not occur because of the ricin injection. Micromeres are indicated by the short branches below each main branch. Micromeres born to the $DM^x$ or $DNOPQ^x$ proteloblasts after ricin injection are not indicated because their birth was dependent upon when the injections were performed.

‡Embryos were scored for A‴-B‴ fusion at 68-82 hours AZD (at least 5 hours after fusion occurs in 100% of control embryos).

The total percent of embryos with A‴-B‴ fusion in each category are in bold. The number of embryos with A‴-B‴ fusion over the total number of embryos scored in each category are in parentheses.

*These data were compiled from more than one clutch of embryos.

§Sketches depict embryos showing the consensus result with lineage tracer (shading) injected into the A lineage. Ricin injection into $DM^x$ or $DNOPQ^x$ or into both late stage $DM^x$ and $DNOPQ^x$ does not block fusion.

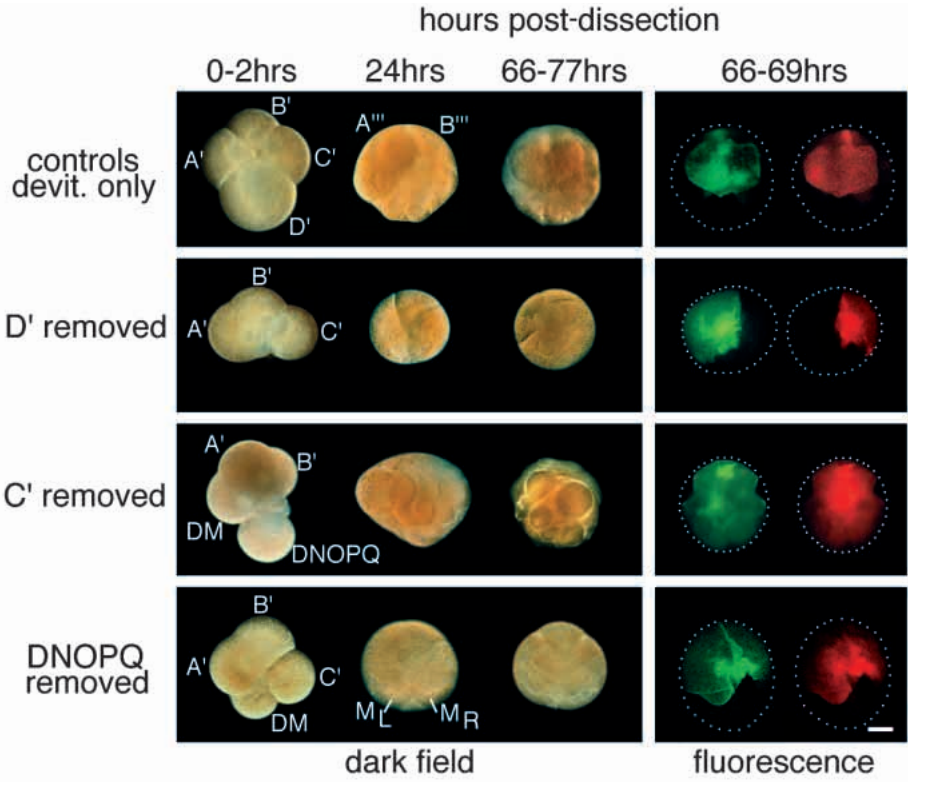



**Fig. 2.** Removal of macromere D′ blocks A‴-B‴ fusion, but removal of cell C′ or DNOPQ does not. (Left panels) Living embryos photographed with dark field illumination either 0-2 hours, 24 hours or 66-77 hours after devitellinization and dissection. In embryos from which C′ was removed, D′ still divided to form DM and DNOPQ within 2 hours after the dissection. By 24 hours post-dissection, unoperated embryos were in stage 7, and teloblasts were present except in the embryo from which D′ had been removed. In embryos from which DNOPQ was removed, the $M_L$ and $M_R$ teloblasts and bandlets formed. By 66-77 hours post-dissection, unoperated embryos were in stage 8. In embryos from which C′ was removed, the A and B quadrant macromeres spread to surround the D quadrant teloblasts, but the teloblasts are more prominent than in control embryos because there are fewer yolky macromeres to obscure them. (Right panels) Epifluorescence photomicrographs or confocal images of comparable embryos fixed 66-69 hours post-dissection. To assay for A‴-B‴ fusion, A or A′ was injected with FDA (green) and B or B′ was injected with RDA (red) in each embryo. The two images in each fluorescence panel are of the same embryo, in the same orientation; dots indicate embryo contours. A‴-B‴ fusion occurred in all embryos except those from which D′ had been removed as evidenced by the fact that FDA and RDA now label identical portions of the embryo. In the embryo from which D′ had been removed, fusion was blocked, so FDA and RDA still occupy distinct domains. Except for the embryo from which C′ was removed (ventral view) all embryos are viewed from the animal pole. Scale bar, 100 μm.

DNOPQ‴], A‴-B‴ fusion occurred in greater than 65% of the embryos (Table 2; Fig. 3C). One interpretation of these results is that there is a critical time window beginning approximately 11 hours AZD during which the inductive effect of D lineage cells becomes resistant to ricin injections.

### Which cells are sufficient to induce fusion

When DM (before it made any micromeres) and also dnopq′ and dnopq″ were arrested, leaving DNOPQ″ unarrested, fusion occurred in 65% (11/17) of the embryos. This is comparable to the 67% of embryos that fused when both $DM^x$ and $DNOPQ^x$ were arrested late, after the birth of their micromeres (Table 2). Therefore, DNOPQ″ and/or its derivatives were sufficient to induce A‴-B‴ fusion in the absence of influence from DM, dm′, dm″, dnopq′ and dnopq″. The result that DNOPQ″ was sufficient to induce fusion complements results obtained from the dissection experiments in which $DNOPQ^x$ was removed (Table 1). The results of those experiments suggest that DM is sufficient to induce A‴-B‴ fusion. But, there are some caveats to those experiments because of the technical problems associated with the dissection technique. The results of ricin injection into only part of the D lineage, however, show unequivocally that biochemical arrest of only part of the D lineage does not block fusion. Independent of whether the mesodermal proteloblast $DM^x$ or the ectodermal proteloblast $DNOPQ^x$ was arrested, and independent of how many micromeres the proteloblast had made by the time of the injection, A‴-B‴ fusion occurred in 71-91% of the embryos (Table 2; Fig. 3D). Moreover, fusion occurred during the same time window as in unperturbed embryos; A‴-B‴ fusion did not occur prior to 47 hours AZD but did occur by 64 hours AZD (data not shown). Similar results were obtained when RNase was used to arrest $DM^x$ (data not shown).

### Biochemical arrest of the fusion partners

We also undertook experiments to determine if fusion was sensitive to biochemical arrest of the fusion partners themselves. For this purpose, we biochemically arrested $A^x$, $B^x$ or both $A^x$ and $B^x$ at varying times by ricin injection and monitored whether or not fusion occurred. Fusion was blocked when either or both $A^x$ and $B^x$ were arrested early (i.e. before the birth of the a‴ and b‴ micromeres at 11 hours 50 minutes AZD; Table 3; Figs 4A, 5). Because arresting either the A or B lineage macromere early in development is sufficient to block fusion, we conclude that $A^x$ and $B^x$ both must play active roles in the fusion process.

To further investigate the roles of $A^x$ and/or $B^x$ during the fusion process, cells $A^x$ and/or $B^x$ were injected with ricin at progressively later times in many different clutches of embryos. These experiments yielded two unexpected results. First, biochemical arrest of $A^x$ and/or $B^x$ is effective at blocking fusion only within a narrow time window. Biochemical arrest of A‴ and/or B‴ after the beginning of stage 5 no longer blocked fusion (Table 3; Figs 4B, 5). This stage, 13 hours 45 minutes AZD, is 37-49 hours before fusion takes place. Second, the A and B lineages have slightly different time courses of ricin sensitivity; $A^x$ becomes resistant to the fusion blocking effects of ricin approximately

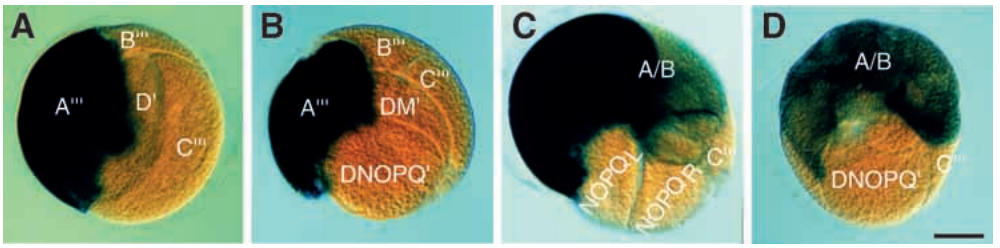



**Fig. 3.** Early biochemical arrest of D quadrant derivatives blocks A‴-B‴ fusion, while later or partial arrest does not. Photomicrographs of embryos fixed and stained for the β-galactosidase reaction product 6-22 hours after A‴-B‴ fusion normally occurs. β-galactosidase was injected into $A^x$ during stages 3-6a and D lineage cell(s) were injected with ricin as described for each panel. Ricin-injected cells underwent at most one further division during the more than 65 hours between the time of injection and the time of fixation. The vitelline envelope is visible as the membranous tissue surrounding each embryo. (A) A‴-B‴ fusion (assayed at 69-85 hours AZD) was blocked when ricin was injected into macromere D′ at stage 4a (7-8 hours AZD), or (B) injected into DM′ and DNOPQ′ at stage 4b (10-11 hours AZD), but (C) not when injected intoDNOPQ‴ and both the left and right M teloblasts at stage 4c (13 hours AZD). This embryo was apparently fixed soon after the onset of fusion as evidenced by the unequilibrated reaction product between the fusing A‴ and B‴ macromeres; also the injected DNOPQ‴ divided into $NOPQ_L$ and $NOPQ_R$ because the injection occurred only minutes before the onset of this division. (D) A‴-B‴ fusion was not blocked when ricin was injected into only DNOPQ′. Scale bar, 100 μm.

2 hours before $B^x$ does. To further test this conclusion, we split three different clutches in half and compared the effects of arresting $A^x$ versus $B^x$. That is, A″ or A‴ was injected with ricin in half the embryos of each clutch while B″ or B‴ was injected in the other half of the embryos. The ricin injections for each embryo of a clutch occurred within 5 minutes of each other and occurred during the transition period from the ricin sensitive to the ricin insensitive block to fusion (Fig. 5). When these embryos were scored for $A^x$-$B^x$ fusion, we found a higher incidence of fusion in the A lineage-arrested versus the B-lineage arrested embryos, confirming that $A^x$ becomes resistant to the fusion blocking effects of ricin earlier than $B^x$ does. Moreover, biochemical arrest of A‴ or B‴ late did not affect the time at which fusion occurred (data not shown).

When ricin was injected into cell $A^x$ and/or $B^x$ before their micromere production was completed, the birth of the subsequent micromeres was blocked. Therefore, one might hypothesize that it is the birth of the micromeres in the A or B lineage that is required for fusion rather than protein synthesis. However, this is not the case for the B lineage since ricin injections into B‴, up to two hours after the birth of b‴, blocked A‴-B‴ fusion in 73% of the embryos (Table 3, B‴early).

This argument can not be used for the A lineage since fusion occurred in 27% of the embryos when ricin was injected into A″ (before a‴ was born) but in 74% of the embryos when ricin was injected into A‴ (within 2 hours after a‴ was born; Table 3). Therefore, to test if a‴ is required for A‴-B‴ fusion, we ablated the a‴ micromere within 15 minutes of its birth by overinjecting it with 0.5 N KCl and 1% fast green until it visibly burst due to the overinjection. After a‴ ablation, A‴-B‴ fusion always occurred (14/14 embryos). Moreover, when a‴ and A‴ were both injected with ricin, fusion occurred (9/10 embryos). Thus, A‴-B‴ fusion does not require the A or B lineage micromeres but presumably does require protein synthesis in both the A and B lineage macromeres during a critical time approximately 11-14 hours AZD (37-52 hours before fusion occurs).

## DISCUSSION

We have previously shown that endodermal precursor cells in embryos of the glossiphoniid leech *Helobdella robusta* fuse in a stepwise manner during discrete and narrow time windows (Liu et al., 1998). The aim of the experiments reported here was to investigate the regulation of the first of these fusions, i.e. to determine whether the fusion proceeds autonomously or is induced.

### Source of the inductive signal

By culturing embryos after dissecting away either the D or C quadrant macromere, we have shown that the fusion of A‴ and B‴ requires the presence of the D lineage but not the C lineage. The same results were obtained using micro-injection techniques to biochemically arrest the D or C quadrant cells instead of dissecting them from the embryo. These results demonstrate ‘third party’ regulation of an embryonic cell fusion.

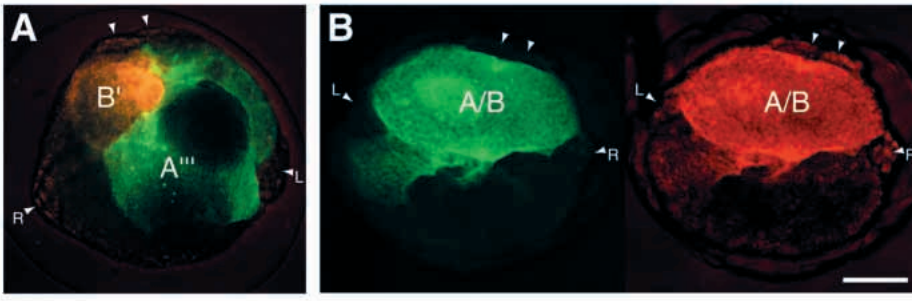



**Fig. 4.** Ricin injection into B′ blocks A‴-B‴ fusion while injection into B‴ does not. Confocal micrographs of embryos in which $B^x$ was injected with ricin and RDA and $A^x$ was injected with FDA. The left (L) and right (R) germinal bands (single arrowheads) and the germinal plate (paired arrowheads at the top of each panel) are indicated in each image. (A) Ricin injection into B′ (~8 hours AZD) caused it to round up and blocked fusion. RDA and FDA occupy distinct regions of the embryo (83 hours AZD; ventral view, anterior up). Note that the rounding up of B′ because of the ricin injection results in formation of a slightly off-center germinal plate. (B) Ricin injection into B‴ (15.5 hours AZD) did not block A‴-B‴ fusion, as evidenced by the appearance of FDA (left image) and RDA (right image) in identical regions of the same embryo (75 hours AZD; dorsal view, anterior up). Scale bar, 100 μm.

**Table 3. Early biochemical arrest of $A^x$, $B^x$, or $A^x$ and $B^x$ prevents fusion while late arrest does not**

| $A^x$ | | Fusion after ricin injection into $B^x$ | | $A^x$ and $B^x$ | |
|---|---|---|---|---|---|
| Cell injected | % Fusion (*n*) | Cell injected | % Fusion (*n*) | Cells injected | % Fusion (*n*) |
| A | 0% (0/42) | B | 2% (1/45) | A+B | 18% (2/11) |
| A′ | 2% (1/44) | B′ | 0% (0/35) | A′+B′ | 17% (1/6) |
| A″ | 27% (14/51) | B″ | 0% (0/29) | | |
| A‴early[1] | 74% (73/99) | B‴early[1] | 27% (50/182) | | |
| A‴late[2] | 91% (91/100) | B‴late[2] | 93% (92/99) | A‴+B‴ | 100% (38/38) |

[1]A‴early and B‴early indicate ricin was injected after the birth of a‴and b‴but before the beginning of stage 5, between 11 hours 50 minutes and 13 hours 40 minutes AZD.

[2]A‴late and B‴late indicate that ricin was injected during stage 5 or early stage 6a, between 13 hours 45 minutes and 18 hours 0 minutes AZD.

Results of our dissection and biochemical arrest experiments also have shown that even a part of the D lineage is sufficient to induce fusion. Specifically, fusion occurs in approximately 50% of the embryos if only cell DNOPQ is removed. Three non-exclusive explanations could account for the reduced and variable incidence of A‴-B‴ fusion in embryos from which DNOPQ has been dissected: (1) experimental trauma may reduce the incidence of fusion; (2) contact with DNOPQ may be required for fusion, but only briefly, so that the timing of the dissection is critical; (3) in the absence of DNOPQ, the signal provided by cell DM may be near threshold. Biochemical arrest experiments in which fusion occurs when either $DNOPQ^x$ or $DM^x$ are arrested suggest that the inductive signal is distributed among D quadrant progeny, consistent with the third possibility.

The question of exactly which D lineage progeny cells are capable of activating fusion remains open. It is clear that the teloblasts and blast cells are not required since A‴-B‴ fusion occurs when $DM^x$ and $DNOPQ^x$ are biochemically arrested before formation of the teloblasts. However, we can not rule out the possibility that these cells may be involved in regulating fusion during normal development.

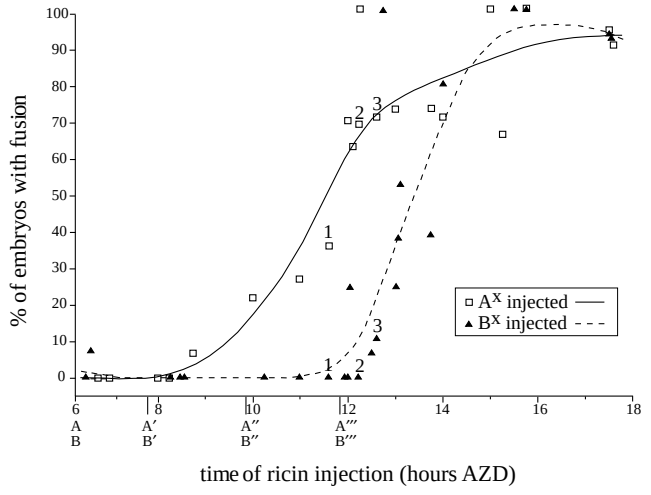



**Fig. 5.** The time course of the ricin-mediated block to fusion differs in the A and B lineages. After ricin injection into $A^x$ or $B^x$, embryos were cultured until at least 77 hours AZD (at least 14 hours after fusion occurs in 100% of control embryos) and then scored for fusion. Each data point represents a single experiment containing 7-36 embryos (avg. 15.4). Numbered data points indicate split clutches in which half of the embryos had $A^x$ arrested while the other half had $B^x$ arrested at the same developmental time ±5 minutes. The x axis indicates the timing of ricin injections (hours AZD), with the divisions of the A and B lineages indicated. Best-fit curves were generated using a generalized additive model.

## Critical periods for the fusion-blocking effects of biochemical arrest

We have also discovered that there is a critical time window, long before the actual fusion event, during which biochemical arrest of the A, B or D lineage prevents fusion. If any one of these lineages is arrested by injection of ricin or RNase after 14 hours AZD, A‴-B‴ fusion proceeds normally at 51-63 hours AZD. However, if these lineages are biochemically arrested before 10 hours AZD, fusion is blocked.

We find that the critical period for the ricin-mediated block to fusion differs between the A and B lineages. Specifically, the A lineage becomes resistant to the fusion-blocking effects of biochemical arrest two hours earlier than does the B lineage. This result was unexpected because $A^x$ and $B^x$ are born simultaneously as sister cells from cell AB and undergo their subsequent divisions in synchrony. As described previously (Liu et al., 1998), cells $A^x$ and $B^x$ differ in that $A^x$ has more extensive contacts with the D quadrant derivatives than does cell $B^x$. Assuming that the inductive signal is transferred from the D quadrant derivatives to $A^x$ and $B^x$ by cell-cell contact, the difference in the timing of the critical period for the ricin-mediated block to fusion may reflect the fact that the A and D quadrants have a more extensive area of contact through which to transmit and receive the inductive signal than do the B and D quadrants.

## Significance of biochemical arrest

Assuming that ricin and RNase cause biochemical arrest by preventing protein synthesis in cells of the leech embryo as in other eukaryotes, our results suggest that A‴-B‴ fusion requires protein synthesis in the A, B and D lineages approximately 12 hours into development, but not during the 40 hours immediately preceding fusion. Thus, de novo protein synthesis in these lineages is most likely not controlling the actual time at which fusion begins. More likely, proteins synthesized during the first 12 hours of development are modified post-translationally at some time during the next 40 hours prior to A‴-B‴ fusion, and it is these modifications that trigger the actual fusion event. Post-translational modifications could alter the cellular localization of a protein, facilitate interactions with other proteins, and/or initiate signaling cascades mediated by second messengers. Indeed, post-translational modifications and the activation of signaling cascades are implicated in the regulation of myoblast fusion (Knudsen, 1991).

The critical time window for required protein synthesis in the D lineage (approx. 11 hours after zygote deposition) is similar to that of the A and B lineage (approx. 12 and approx. 14 hours after zygote deposition, respectively). All three of these time points are during stage 4b. Bissen and Weisblat (1991) have shown that this is also the time at which the normal cleavage divisions become sensitive to the transcriptional inhibitor α-amanitin, suggesting that the transition from maternal to zygotic control of development begins during this stage as well.

The results of our biochemical arrests of $A^x$ or $B^x$ also indicate that A‴-B‴ fusion requires the active participation of both A‴ and B‴. Biochemical arrest of either one of these lineages during the early critical period blocked fusion. The requirement for activity in both of the fusion partners is similar to fusion of myoblasts during muscle formation and the fusion of sperm and egg during fertilization. The observation of paired vesicles lining the apposing membranes just prior to myoblast fusion in *Drosophila* suggests a bi-directional component to myoblast fusion (Doberstein et al., 1997). The fact that myoblasts must recognize and adhere to one another prior to fusion also suggests that both cells must actively participate in fusion.

Biochemical arrest experiments also suggest that protein synthesis is required for A‴-B‴ fusion and also for its regulation by D quadrant derivatives. But they do not prove that proteins are directly involved in the signalling or, if so whether membrane bound or secreted factors act as the inducing agent. Nor do they address the question of when the putative proteins are acting, either in terms of when the D lineage signals the $A^x$ and/or $B^x$ cells or when $A^x$ and $B^x$ become competent to fuse. It is therefore unknown whether the D lineage signals $A^x$ and/or $B^x$ early in development (i.e. during the critical time period approximately 40 hours prior to fusion) or later, just before fusion. Moreover, the D lineage signal might be required over an extended period of time.

### Comparison with other animals

Embryonic yolk cells are a common feature in many different taxa and are presumed to have arisen independently many times during evolution. Recent work in molecular phylogeny has led to the suggestion that the protostomes contained two distinct groups even before the Cambrian period (Aguinaldo, 1997; Balavoine and Adoutte, 1998). Arthropods and nematodes are assigned to the Ecdysozoa, while annelids and molluscs are assigned to the Lophotrochozoa.

In the nematode *C. elegans*, cell fusions that occur in the formation of various epithelia are predictable with respect to the identity of the fusion partners and the timing of the fusion (Podbilewicz and White, 1994). Within the ventral hypoderm, cells are recruited to fuse with a pre-existing syncytium by signals emanating from the syncytium itself (Herman and Hedgecock, 1990), but whether examples of 'third party' inductive regulation of fusion occurs in nematode remains to be determined.

SYCs are present in both annelids and arthropods, and examples of SYC formation by cell fusion have been reported for both groups as well (Anderson, 1973; Liu et al., 1998). Thus, the possibility exists that similar processes of regulated cell fusion await discovery in arthropods. As far as we know, there have been no analyses in molluscs that address the possibility of SYC existence and no reports of cell fusion other than fertilization. It will be interesting to determine if the regulated SYC formation we have described in leech is a common feature in these taxa or others.

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