Selective Disruption of Gap Junctional Communication Interferes with a Patterning Process in Hydra

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The cells that make up the body column of hydra are extensively joined by gap junctions, capable of mediating the rapid exchange of small hydrophilic molecules between the cytoplasms of neighboring cells. Both the rate of transfer of small molecules through the gap junctions and the rate of return of gap junction coupling after grafting experiments are sufficiently rapid to mediate events in the patterning of hydra tissue. Antibodies to the major rat liver gap junction protein (27,000 daltons) recognize a gap junction antigen in hydra and are effective in eliminating junctional communication between hydra cells. The antibodies perturb the head inhibition gradient in grafting operations, suggesting that cell-cell communication via gap junctions is important in this defined tissue patterning process.

A LARGE BODY OF EXPERIMENTAL AND THEORETICAL WORK indicates that interactions between neighboring cells play a major role in the patterning of tissues both during development and regeneration (*1*). The range and timing of some of these interactions are consistent with the diffusional exchange of small messenger molecules (*2*). Gap junctions, which can mediate the direct exchange of molecules up to about 1 kD between the cytoplasms of neighboring cells (*3, 4*), are widely distributed in developing tissues (*5*), and it has been proposed that they may play an important role in tissue patterning (*6*). Although this proposal remains popular, few experiments, other than those demonstrating patterns in the passage of indicator dyes from cell to cell, have been performed to experimentally test the role of junctional communication in developmental processes.

In order to elucidate the proposed role of gap junctions in developing tissues, we have examined patterning of a freshwater coelenterate, hydra. Hydra has been studied extensively to gain insight into the interactions that are important in patterning (*7*). The simple body plan, with a head and a foot at opposite ends of a hollow, two-layered, epithelial cylinder, permits analyses and grafting operations that would be difficult, if not impossible, in other species. Such grafting experiments indicate that the body plan is reestablished rapidly after experimental intervention. A small fragment excised from any part of the hydra body column is capable of repatterning into a complete miniature version of the animal. The original apical end of the fragment always forms the head in the repatterned animal, and the original basal end forms the foot. This form of assembly implies that the cells of the body column have some inherent positional information, and that interactions between the cells in regenerating fragments determine their final pattern.

Grafting experiments, in which fragments of hydra tissue are juxtaposed in novel arrangements, indicate that two developmental gradients play a role in establishing the position of a regenerating head. One, the head activation gradient, is due to a relatively stable property of the tissue. Head activation is distributed monotonically, with tissue at the upper end of the body column having a greater ability to form a head than the tissue further down the body column (*8*). The other gradient, the head inhibition gradient, is also monotonically distributed, but is very different in character. Head inhibition originates from an established head and is transmitted down the body column, preventing the tissue from forming another head. This property is labile because head inhibition in the body column decreases with a half-life of 2 to 3 hours after removal of the head (*9*). The phenomenon of head inhibition is commonly assumed to be due to the steady-state production of diffusible "head inhibitor" by the head and its degradation in the tissues through which it diffuses. This concept of a diffusible patterning substance was originally proposed by Wolpert (*10*), and was later incorporated into reaction-diffusion models by Gierer and Meinhardt (*11*) and MacWilliams (*12*). The ability of these models to fit the experimental results on head inhibition lends support to these proposals (*9*). Consistent with the idea of a diffusible substance, careful studies of the head inhibition gradient following grafting or temperature shifts indicate that the establishment of a new steady state requires 4 to 6 hours (*9*). This is about the time expected to reestablish a gradient by the diffusion of a small molecule along the length of the hydra. Furthermore, an activity has been isolated from hydra with some of the qualities and behaviors expected of a head inhibitor (*13*). The molecule (or molecules) responsible for the inhibition activity has yet to be fully characterized but appears to be a hydrophilic nonpeptide with a molecular size of 500 daltons or less.

The idea of a diffusible gradient through the tissue of the body column has led to the proposal that communication through gap junctions might be required for the transmission of head inhibition from the head down through the epithelial cells of the body column (*14*). Electron microscopy has shown that the epithelial cells of the

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hydra body column are extensively linked to one another by gap junctions (*15*), and several lines of circumstantial evidence implicate a role for gap junctions in the patterning of the body column. First, experiments with intracellular microelectrodes indicate that the cells are both dye and electrically coupled, demonstrating the presence of a functional communication pathway between the cells (*14, 16*). Second, analysis of a mutant with an altered head inhibition gradient indicates that the effect of the mutation is associated with the epithelial cells of the body column (*17*). It is through the epithelial cells that the gap junctions would be expected to communicate diffusible substances. Finally, the putative head inhibitor isolated from hydra (*13*) is both hydrophilic and small enough to pass rapidly from cell to cell through the gap junction channels.

In this article, the possible role of gap junction communication in the patterning of the hydra body plan is examined. Evidence is presented to demonstrate that the rate of both the diffusion of tracer dyes through the gap junctions and the return of cell-cell communication after a grafting operation are sufficiently rapid to mediate the head inhibition gradient. Then, the potential relation of gap junction communication to the transmission of head inhibition down the body column is examined with the use of an antibody to the gap junction protein.

Dye transfer and electrical coupling in hydra. Our study extends and refines previous observations that the epithelial cells of the hydra body column are dye-coupled and electrically coupled to one another (*14, 16*). Body columns of *Hydra attenuata* were threaded onto glass capillary tubing, and an epithelial cell was impaled with a micropipette containing the small hydrophilic fluorescent dye Lucifer yellow. Dye was iontophoretically injected with current pulses, and the membrane potential was monitored continuously between the current pulses to determine the location of the electrode tip in the cell. The membrane potential of the cytoplasm was negative (-12 ± 5 mV, standard deviation), whereas the potential of the large central vacuole of the epithelial cells was positive ($+20 \pm 9$ mV).

Dye passage between the epithelial cells was tested in 25 animals, of which 23 showed passage of the dye to neighbors. An example of cell to cell transfer of Lucifer yellow is shown in Fig. 1A. The two cases that did not demonstrate dye passage had a single, brightly filled vacuole in a single cell with no detectable dye in the cytoplasm of the cell. This confirms our previous findings of coupling in the body column of hydra and of the apparent lack of coupling between cells when the dye is predominantly injected into the vacuoles (*14, 16*). To control for possible passage of dye along the tissue through the interstitial spaces outside the cells, dye injections were purposely made into the interstitial space. This produced a distinctly different pattern of dye spread, with only fine lines of fluorescence appearing between the cells. Furthermore, when viewing the epithelium tangentially, we observed that the intracellular passage of the dye reached the outermost (apical) margins of the cells, whereas extracellular passage was restricted to the basolateral spaces between cells.

The high percentage of cases (92 percent) demonstrating dye coupling contrasts with our earlier study (*14*) in which only about 50 percent of the dye-injected cells appeared to be dye-coupled when viewed 5 to 15 minutes after injection. It is now apparent that the lower fraction of noticeable passage was due to the strong coupling between the cells, the small amount of dye injected (about 40 times less than in our study), and the lapse of several minutes between the dye injection and observation. To confirm this, we injected a small amount of dye into a hydra cell while observing the distribution of the dye. In a few seconds, the dye had passed to several neighbors up to four cell diameters away. The dye injection was then terminated, permitting the injected dye to continue passing to other cells, and within 2 to 5 minutes the dye was no longer detectable, except in the injected cell and sometimes in its immediate neighbors. Thus, in our previous study, the absence of coupling in 50 percent of the successful injections was probably not due to lack of dye coupling, but rather to the presence of very strong coupling.

Electrical coupling between the cells of the hydra body column was examined to provide another assay of cell-cell communication. One electrode was placed in an epithelial cell and another electrode in a second epithelial cell about five cell diameters away. Injection of

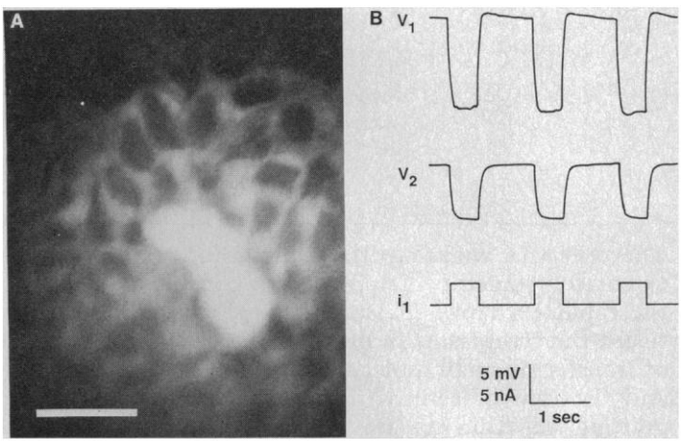


Fig. 1. Coupling in hydra. (**A**) Fluorescence image of dye coupling about 1 minute after injection of Lucifer yellow into an epithelial cell. Intracellular dye shows a characteristic pattern with bright areas corresponding to the cytoplasms of the cells and dark central regions corresponding to the large central vacuoles. (**B**) Electrical coupling between the hydra epithelial cells. Voltage deflections in the cell injected with current (i_1, V_1) and a neighboring cell (V_2) indicate a coupling coefficient of 0.6 in this example (scale bar, 100 μm). Experiments were performed on *Hydra attenuata*, maintained and operated on as described previously (*17*) in hydra medium (HM, 1 m*M* $CaCl_2$, 1 m*M* $NaHCO_3$, and 10 μ*M* EDTA). Food was withheld for at least 2 days before an experiment. For dye-coupling or electrical-coupling measurements, the head and the foot of the hydra were removed and the remaining body column was then strung onto a 0.4-mm (outside diameter) glass microcapillary. The glass capillary was secured with dental wax in the bottom of a 35-mm petri dish containing HM. Tissue treated in this manner remained healthy for hours if left strung on the glass capillary tubing. Ten minutes before dye or electrical coupling was to be measured, the HM bathing the animal was exchanged with a solution of the anesthetic Nembutal (4 mg/ml) in HM. The Nembutal greatly reduced the spontaneous movements of the animal, thereby permitting more stable impalement of the hydra cells with intracellular electrodes and simplifying the photography of the dye-passage patterns. The tissue remained healthy in HM-Nembutal for 1 hour. Some experiments were performed without the Nembutal to test for adverse effects of the drug, and no differences in either the spread of injected dye or electrical coupling were noted. The dye injection micropipettes were pulled from thin-walled aluminosilicate capillary glass (1.2 mm; Haer Ultratip glass), the tips were backfilled with 20 percent Lucifer yellow (Molecular Probes) in 100 m*M* LiCl, and the shank of the electrode was then backfilled with 1.2*M* LiCl. Electrodes (resistance, 65 Mohm) were connected to a current-passing microelectrode amplifier (NeuroData Instruments). Successful impalement of the cell was monitored by the membrane potential, and the dye was transferred by iontophoresis into the cell with 250-msec, 2-Hz hyperpolarizing current pulses of 2- to 4-nA amplitude for 2 to 4 minutes. During dye injection, the animal was protected from light to minimize any phototoxic effects of the dye. Data were recorded on high-speed slide film (Ektachrome 400) or on videotape (SIT image intensifying camera). Electrical coupling experiments were performed as described above except that two KCl-filled micropipettes were used to record from two nearby cells. A bridge-type amplifier (Getting) was used to inject current and record the voltage deflection produced in that same cell. A second, voltage-follower type amplifier was used to record the voltage deflection in a nearby cell (approximately five cell diameters away); a virtual ground was used to measure the injected current. The injected current and the resultant voltage deflections were recorded on a strip-chart recorder and an FM tape recorder for later analysis. The coupling coefficient, the ratio of the voltage response in the neighboring cell to the voltage response in the injected cell, was used as a measure of the strength of electrical coupling between the cells.

current through one of the electrodes led to a voltage deflection in the second cell, indicating the presence of electrical coupling (Fig. 1B). The coupling coefficients (the ratio of the voltage in the neighbor to the voltage in the injected cell) ranged from 0.2 to more than 0.7 (n = 25). Some variability in the coupling coefficient was observed during recording from the same pair of cells. This variability may be due to the fact that the impaled cells are part of an excitable tissue, and that the light anesthesia used for these studies diminished, but did not abolish, the neural control of the tissue. Because the coupling coefficient is a function of the conductances of both the gap junctions and the nonjunctional membrane, the changes in nonjunctional membrane conductance produced by synaptic activity would be expected to alter the coupling coefficient.

Rates of dye movement and reestablishment of coupling. If gap junctions are important in the head inhibition gradient, both the rate of dye passage and the reestablishment of gap junctional communication between cells at a graft border must be sufficiently rapid to fit the known dynamics of head inhibition in transplantation experiments. Grafting analyses show that the head inhibition gradient can re-form in 4 to 6 hours (*9, 12*).

The cell-cell passage of injected dye can be used to estimate the time required for diffusion of a small molecule along the length of the body column. With our experimental apparatus and because of the rapid passage of dye from cell to cell, simple timing of the spread of dye to near neighbors cannot be used to obtain an accurate measure of the rate of dye spread. Therefore, a point 1 mm away from the injection site was chosen, and the time required for the dye fluorescence to reach this distant site (with an intensity of about 50 percent of that at the injection site) was determined. Since diffusion in the epithelial cells over this distance is largely in two dimensions, the simple equation $D = x^2/4t$ can be used to estimate the diffusion constant of Lucifer yellow through the tissue (where x is 1 mm; D is the diffusion constant, and t is the time in seconds). In all 15 hydra examined, the dye had moved the 1-mm distance within 15 minutes, yielding an estimate of 3×10^{-6} cm^2/sec for the diffusion coefficient. This diffusion rate is consistent with the rate of spread of head inhibition predicted from grafting experiments by MacWilliams (*9*) and with models proposed for the patterning of hydra (*10–12*).

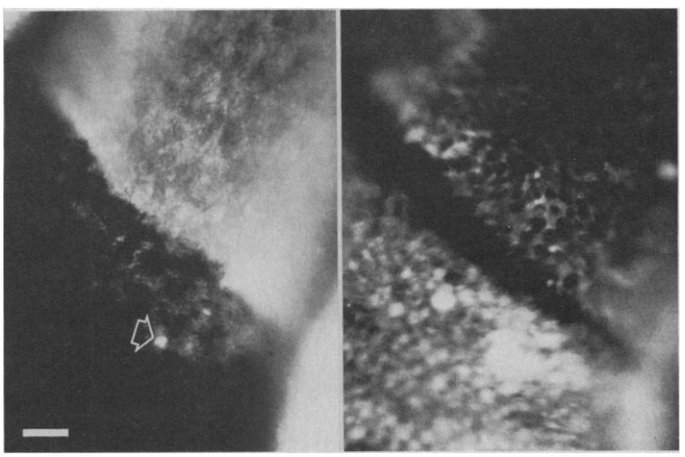
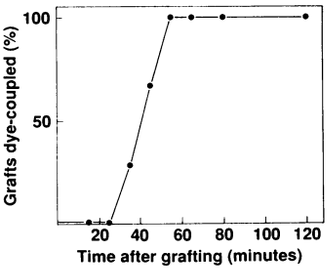


Fig. 2. Dye coupling between grafted fragments returns within 1 hour. (**A**) Combined fluorescence and bright-field image of a graft between carbon-marked and unmarked tissue 1 hour after grafting. The carbon-marked tissue is to the lower left, and the unmarked tissue is to the upper right. The site of dye injection (arrow) can be recognized by the brightly labeled vacuole in the injected cell. (**B**) Fluorescence image showing passage of the dye to neighboring cells and to cells across the graft boundary. Cells on both sides of the graft border show the typical pattern of a bright ring of dye-filled cytoplasm surrounding a dark central vacuole. The dark band at the center is due to carbon particles that build up at the graft border (scale bar, 100 μm). Two cylindrical fragments of hydra, one marked with carbon particles (by injection of india ink into the gut the day before) were grafted together on a 0.4-mm glass capillary. Small sleeves of polyethylene tubing with an inner diameter slightly larger than the glass tubing were used to hold the tissue fragments in contact. Animals were labeled with carbon particles by injecting their gastric cavities with biological india ink (Pelikan) one day before the grafting experiment. The carbon particles were ingested by the endodermal cells and remained clearly visible as a cell marker for several days (*18*).

Fig. 3. Fraction of grafts dye-coupled as a function of time after operation. For each data point, 5 to 12 grafts were examined.



To define the time required for dye and electrical coupling to return following a grafting operation, we grafted together two rings of tissue excised from the body column, and the dye passage between them was tested at various times. One of the two rings was labeled with carbon particles (india ink) (*18*) so that the graft border would remain clearly identifiable. The time course for the return of coupling was assayed in a double-blind experiment, in which one experimenter performed the grafts and gave them at varying times to another investigator, who then determined if coupling had returned. The results indicated that dye coupling was initially detectable about 40 to 50 minutes after a grafting operation (Fig. 2), with all grafts demonstrating coupling by 1 hour (Fig. 3). Electrical coupling measurements performed in parallel (n = 20) demonstrated that electrical coupling returned within 50 minutes. This rate of return is sufficiently rapid for gap junctions to participate in maintaining the head inhibition gradient.

Gap junction antibodies. To determine if gap junction mediated communication plays a role in the transmission of head inhibition, some means of reversibly interfering with this communication pathway is necessary. Our experimental design is based, in part, on the work of Warner *et al.* (*19*), which demonstrated the feasibility of using antibodies to the gap junction protein to disrupt cell-cell communication in amphibian embryos.

Two affinity-purified rabbit polyclonal antibodies were used in these experiments. We were able to generate both reagents (termed A and B) by immunizing rabbits with the major protein present in rat liver gap junctions, a polypeptide of 27,000 kilodaltons. Protein from isolated rat liver gap junctions (*20*) was electroeluted from polyacrylamide gels for immunization (*19*). The sera from the immunized animals were purified on an affinity column containing the eluted 27-kD protein conjugated to Sepharose 4B. The resulting affinity-purified reagents have been shown to bind to the cytoplasmic surfaces of intact rat liver gap junctions, and they bind specifically to homologous antigens (27- and 54-kD proteins) in homogenates of rat liver and hydra (Fig. 4), as well as from a variety of species from a range of phyla (*21*). The 54-kD antigen represents a dimer of the gap junction protein which is observed on 12.5 percent polyacrylamide gels. It is equivalent to other dimeric forms of the protein (47 kD and 64 kD) which are observed under different sodium dodecyl sulfate–polyacrylamide gel conditions (*21*). The same antibodies have also been used to disrupt cell-cell communication after intracellular microinjection into embryos of *Xenopus laevis* (*19*); thus, these reagents can be used for both immunochemical and biological studies on gap junctional antigens in different animal species. In hydra, antibody B shows greater affinity to the gap junction protein than the other reagent (antibody

A) by immunoblotting (Fig. 4). In all our experiments, immunoglobulins isolated from the relevant preimmune serum were used as controls.

Indirect immunofluorescence was used to localize the antigen in hydra. The in situ distribution of antigen was examined in whole mounts of fixed hydra, and found to be strikingly similar to that expected for gap junction structures in this organism (Fig. 5). Previous electron microscopic studies have demonstrated that most of the gap junctions are present primarily in either the apical portion of the lateral membrane of the epithelial cells or along the muscle processes at the base of the cells (*15*). In whole mounts, the antigen was localized to both the lateral surface (Fig. 5) and the basal processes. The whole mounts were used because they permit a straightforward localization of labeled structures and they have been used extensively to characterize other antigens in hydra (*22*).

Effects of antibodies on junctional communication. In initial experiments, the ability of antibodies against the 27-kD rat liver protein to block gap junction mediated communication between cells was determined by microinjecting them into hydra epithelial cells and later assaying the transfer of fluorescent dyes between the injected cell and its neighbors. Either antibody A or immunoglobulin (IgG) (1.5 mg/ml) from preimmune animals was co-injected with Texas Red dextran (10 kD, 10 mg/ml, Molecular Probes) under pressure and with conventional intracellular micropipettes. The fluorescent dextran served as a nondeleterious marker of the injected cell since it cannot move out of the cell through either the plasma membrane or gap junction channels (*23*). One hour later, we determined the effect of the injected antibody by iontophoresis of Lucifer yellow into either the antibody-injected cell or one of its neighbors. The Lucifer yellow passed freely into and out of cells injected with preimmune IgG; in contrast, the dye did not enter or leave cells injected with antibody A. Extracellular application of the antibodies had no noticeable effects. These results demonstrate that microinjected antibodies are capable of disrupting junctional communication in hydra.

An experimental design that requires microinjection of antibody into many individual cells of hydra is not practical for developmental experiments. Hundreds of successful injections would be required for each experimental animal. Therefore, it was necessary to develop a procedure that would permit the delivery of antibody to all cells of the organism. For this purpose, a permeabilizing agent, dimethyl sulfoxide (DMSO), was used to introduce or "load" the antibody into the cytoplasm of hydra cells. The animals were treated with antibody at 0.4 mg/ml in 5 percent DMSO in hydra medium at 4°C for 30 minutes, and then allowed to recover for 15 minutes in 4°C hydra medium. Animals observed after either indirect immunofluorescence after fixation, or loading with fluorescently tagged antibodies were dimly fluorescent, confirming that the technique reliably introduced antibodies (rabbit immunoglobulins) into the cells of hydra. After this treatment, the hydra remained healthy. Those exposed to preimmune reagents were indistinguishable from normal untreated animals. The animals exposed to immune reagents A or B were somewhat uncoordinated, demonstrating slower movements and small focal contractions of the body column. This behavior seems consistent with the disruption of electrical coupling between the contractile cells that make up the body column.

Table 1. Treatment with antibody A or B results in an increased incidence of secondary axis formation.

Treatment of host	Grafts (No.)	Secondary axes (No.)	Fraction with secondary axes (%)
Untreated	182	20	11
Decapitated	116	93	80
Antibody A	209	46	22
Antibody B	134	29	22
Preimmune IgG	205	14	7

Dye transfer between the ectodermal cells of the hydra body column was assayed by intracellular injection of either Lucifer yellow or fluorescein complexon (*24*) into an ectodermal cell. The geometry of the hydra ectodermal cells makes it impossible to definitively score passage of dye to one or two neighbors; therefore, passage of the dye more than three cell diameters was used as the criterion for the presence of dye passage. Cells of hydra loaded with either gap junction antibody A ($n = 72$) or antibody B ($n = 48$) remained uncoupled for many hours; whereas, cells in all of the hydra treated with preimmune serum remained coupled ($n = 34$) (Fig. 6). Antibodies A and B were effective for different periods of time, perhaps reflecting their different affinities for the junction antigen in immunoblots. When scored between 1 and 7 hours after loading, 100 percent of the hydra were uncoupled by reagent A

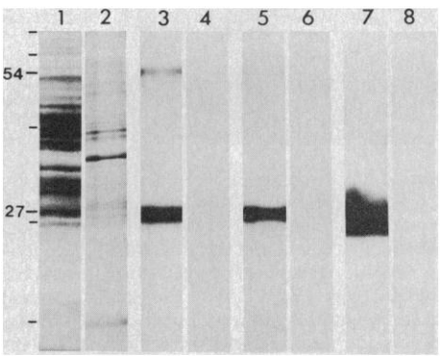


Fig. 4. Immunoblot analysis of the interaction of gap junction 27-kD protein antibodies with rat liver and *Hydra attenuata* homogenates. Lanes 1 (rat liver) and 2 (hydra) show Coomassie blue stained profiles after electrophoresis on polyacrylamide gels. Identical lanes (lanes 3 to 8) were transferred to nitrocellulose paper for treatment with immune and preimmune reagents; bound immunoglobulin was detected by autoradiography after incubation with ^{125}I-labeled protein A. Lane 3 shows the interaction of immune antibody A with a rat liver homogenate; the antibody recognized 27-kD and 54-kD proteins. No antigens were detected by the preimmune IgG (lane 4). Antibody B binds the same two antigens in similarly prepared rat liver samples. Lanes 5 and 7 show the interaction of immune antibodies A and B, respectively, with hydra homogenates. Again, 27-kD and 54-kD proteins are recognized by the immune antibodies and none by the relevant preimmune reagents (lanes 6 and 8). Antibody B (lane 7) shows greater affinity to the antigens than does antibody A (lane 5) when applied at the same concentration and under parallel experimental conditions. Molecular size standards indicated to the left are (from top to bottom) 68-kD (bovine serum albumin), 57.5-kD (catalase), 43-kD (ovalbumin), 25.7-kD (chymotrypsinogen), and 21.5-kD (soybean trypsin inhibitor). Rat liver and *Hydra attenuata* (whole animal) homogenates were prepared by freezing in liquid nitrogen and grinding with a mortar and pestle while frozen. After evaporation of the nitrogen, the powdered frozen tissue was transferred to SDS sample buffer (*29*) where it was solubilized as it thawed. Samples were centrifuged for 5 minutes in an Eppendorf centrifuge and were analyzed in 12.5 percent SDS-polyacrylamide gels. The gels were stained with Coomassie blue (2.5 percent Coomassie blue (w/v), 50 percent methanol, 7 percent acetic acid) for 15 to 20 minutes and then destained (40 percent methanol, 7 percent acetic acid) before viewing. For immunoblots, gels were electrophoretically transferred (*30*) to nitrocellulose paper (BA85, Schleicher & Schuell) which was subsequently "blocked" with 3 percent bovine serum albumin in tris-buffered saline (TBS) for 3 to 4 hours at room temperature to reduce nonspecific binding of the antibody. The blocking solution was then replaced with the appropriate antibody at a dilution of 1:250 in TBS (initial concentration prior to dilution in all cases was 1.5 mg/ml) and the blot was incubated overnight at 4°C. The blot was washed with TBS several times and treated with ^{125}I-labeled protein A (Amersham, >30 Ci/g) for 2 to 3 hours at room temperature. It was then washed twice in TBS containing 0.1 percent Nonidet P-40, in several changes of TBS alone, and finally placed on Kodak XAR (overnight) or Chronex (several days) film with an intensifying screen at −70°C.

(n = 46) and reagent B (n = 29). Animals treated with antibody A were weakly coupled (7/12 cases) or strongly coupled (5/12) when analyzed 8 to 12 hours after treatment, and 100 percent of the cases were coupled when assayed at 13 to 18 hours (n = 8) or 18 to 24 hours (n = 6). Animals treated with antibody B were uncoupled at 8 to 12 hours (n = 9), weakly coupled at 13 to 18 hours (n = 4), and coupling had fully returned by 18 to 24 hours (n = 6). In preliminary electrical coupling experiments, both reagents A and B disrupted electrical coupling, whereas preimmune antibodies had no detectable effect.

Grafting experiments. The grafting operations used in our experiments (Fig. 7) are modified from the grafting experiments used to demonstrate the ability of the head to inhibit the formation of a secondary head (*9*). A small ring of tissue, excised from the apical end of the body column, was grafted midway between the head and the bud of a host animal. In such a graft in normal animals, the head inhibition of the host suppressed the formation of a secondary axis in a majority of the cases, and the grafted tissue was eventually resorbed into the column (Fig. 8A). In a small number of the cases (about 10 percent), the implant formed a head and induced part of the host body column to form a secondary axis (Fig. 8B). In contrast, if the head of the host was removed, the implant was no longer inhibited from developing a head and formed a secondary axis in the majority of the cases (70 to 90 percent). The formation of the secondary axis requires 3 or 4 days, but the commitment to form or not form a second head takes place within the first day (*8*).

To test if gap junctional communication is involved in the head inhibition process, we modified the grafting paradigm such that untreated tissue was grafted to an antibody-treated host (Fig. 7). The antibody should greatly reduce gap junction mediated communication for about 8 hours, a significant fraction of the time involved in the commitment to form a secondary axis (*8, 25*). If head inhibition is transmitted along the body column through gap junctions, disruption of gap junction communication with antibodies should diminish head inhibition at the graft site and thereby increase the incidence of secondary axes. This experimental design has the distinct advantage that the perturbation (blockage of the inhibition signal) leads to a positive result (the formation of a second head) by untreated tissue, thereby minimizing concerns about side effects of the reagents. Control grafting operations consisted of (i) grafts to preimmune treated animals, and (ii) grafts to untreated intact and decapitated animals.

Altogether, 846 grafts were performed in six different experimental sessions. A summary of the results of all 846 grafts is shown in Table 1. In each case, the percentage of the animals in which the implant developed into a full head (hypostome and two to five tentacles; termed a "secondary axis") was determined. Grafts to untreated animals resulted in the formation of secondary axes in only 11 percent of the cases, while 80 percent of the grafts to decapitated animals formed secondary axes. Preimmune treatment of the host animals showed a small but insignificant decrease in the fraction of secondary axes to 7 percent [$P = 0.1$, by a test of differences between proportions (*26*)]. Immune treatment of the host animals increased the incidence of secondary axes to 22 percent, which is statistically significant when compared to both the preimmune treated ($P < 0.002$, with Bonferroni correction) and untreated ($P < 0.01$) grafts.

Gap junctions and patterning. Our data show that the epithelial cells of the hydra body column are strongly dye-coupled to one

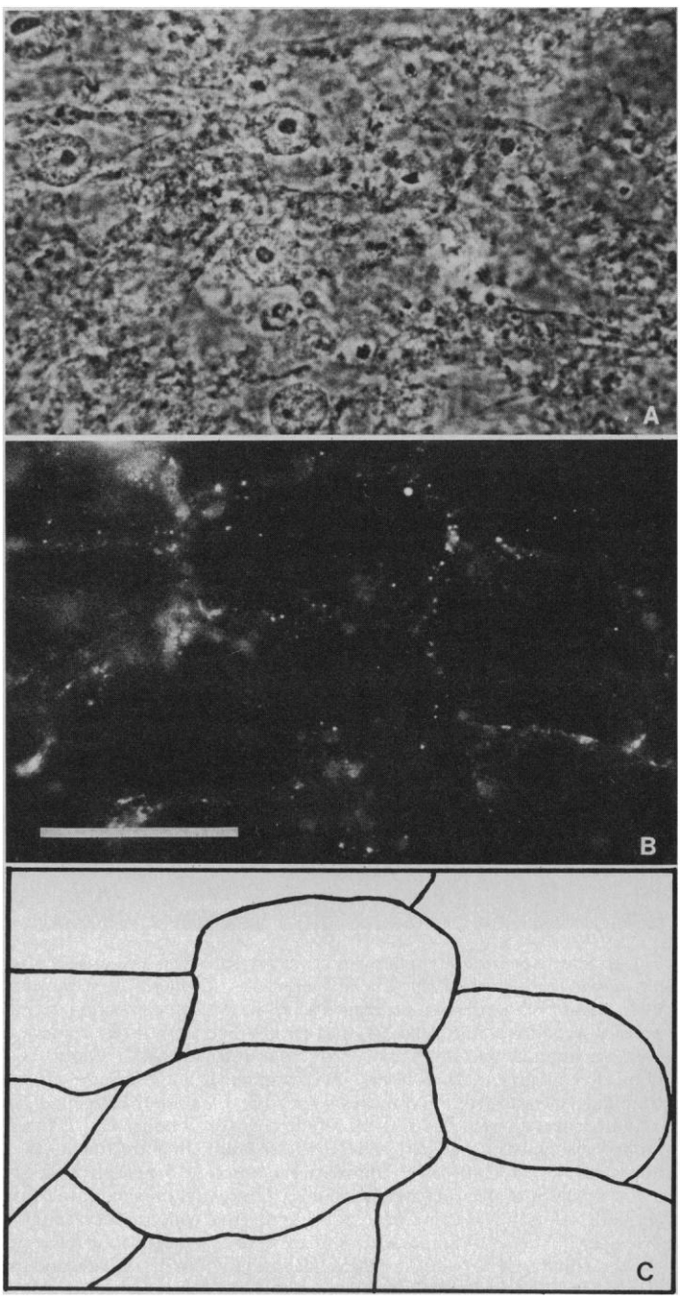


Fig. 5. Indirect immunofluorescence localization of gap junction antibody in *Hydra attenuata*. (**A**) Phase and (**B**) fluorescent views of the same portion of whole-mounted hydra tissue. (**C**) A line drawing of some of the ectoderm cell boundaries to help in interpreting the pattern of fluorescent labeling. The punctate fluorescent pattern is the result of specific binding of the affinity-purified antibody (immune reagent A) to the lateral plasma membranes of contiguous epithelial cells, a region shown by electron microscopy to be rich in gap junctions (*15*). The more diffuse fluorescent regions are from similar punctate fluorescence in a deeper focal plane of the whole mount. In parallel experiments with preimmune reagents, no fluorescence was detectable (scale bar, 50 μm). Indirect immunofluorescence was carried out on whole-mounted hydra with a modification of the technique of Dunne *et al.* (*22*). Whole *Hydra attenuata* were fixed for 45 minutes in Lavdowsky's fixative (10 ml of 37 percent formaldehyde, 50 ml ethanol, 4 ml of acetic acid, and 40 ml of water) and then rinsed with 0.05*M* lysine in 0.1*M* phosphate buffer (four changes over a 45-minute period). They were then rinsed twice for 10 minutes in phosphate-buffered saline (PBS) (*p*H 7.4) and incubated for 45 minutes in primary antibody (immune or preimmune; 1:15 dilution in PBS). After three 10-minute washings in PBS, the animals were "blocked" by treatment with 1 percent bovine serum albumin in PBS for 1 hour and incubated for 45 minutes with FITC-labeled goat antiserum to rabbit immunoglobulins (Miles). They were then washed extensively in PBS and mounted in 0.1 percent phenylenediamine in 90 percent glycerol in PBS, prior to viewing with a Zeiss IM35 photomicroscope equipped for epifluorescence. The entire protocol was carried out at room temperature.

another, as required if gap junctions are to play a role in tissue patterning. Both the time for the coupling to return and the rate of passage of the dye along the body column are rapid. The measured diffusion constant would permit diffusible signaling to pass along the length of a 5-mm long hydra in about 5½ hours. Experiments and models have indicated times that range from 4 to 6 hours for the flow of patterning information along the body column of hydra of this size (*9, 12*). Thus, the gap junctions linking the epithelial cells could provide a sufficient, and logical, pathway for the flow of developmental signals during the patterning of hydra.

The antibody-treated animals showed a significant increase in the percentage of secondary axes formed (7 compared to 22 percent), but this increase did not reach that of the decapitated control animals (22 compared to 80 percent). The smaller fraction of the antibody-treated animals that formed a secondary axis may result from any of several factors. First, the antibody-induced uncoupling of the cells was temporary, lasting only 6 to 12 hours; in contrast decapitation eliminates head inhibition for at least 24 hours (*9*). A grafting experiment in which the head was removed for 6 to 12 hours and then replaced was performed to mimic this shorter-lived elimination of head inhibition, and resulted in only about 50 percent of the animals forming secondary axes (*25*). Thus, a more accurate comparison is between 22 and 50 percent. Treating with the antibody a second time is impractical since this would expose both the donor and the host tissue to the reagents. Second, recent evidence indicates that the endoderm may play an important role in the transmission of head inhibition (*27*). Coupling measurements are difficult to perform on the endoderm because of the overlying ectoderm. While it was possible in a few cases to convincingly show that the endoderm was uncoupled by the antibody, it remains

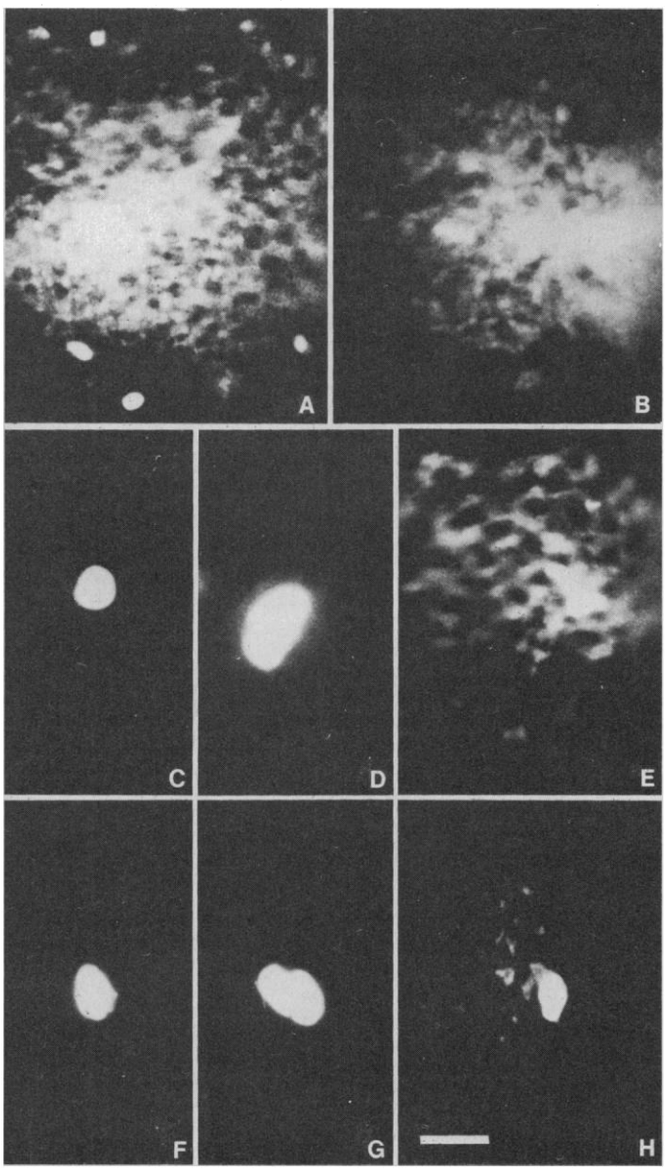


Fig. 6. Effects of antibody treatment on dye transfer between cells of *Hydra attenuata*. Transfer of the dye Lucifer yellow was not altered by treatment of hydra tissue with preimmune reagents, as shown by comparing the dye transfer in control, untreated (**A**) and preimmune treated (**B**) animals. In contrast, immune reagent A abolished dye transfer for up to 8 hours (**C**, 1 hour after treatment; **D**, 5 hours after treatment), but had little effect on coupling when assayed several hours later (**E**, 14 hours). Reagent B also inhibited dye transfer but was more effective (**F**, 1 hour; **G**, 12 hours), limited dye transfer was detected 13 to 18 hours after treatment (**H**, 18 hours) (scale bar, 100 μm). Animals were rinsed in 5 percent DMSO in hydra medium at 4°C and pipetted into a 10-μl well of a multiwell plate; antibody solution was then added to bring the final concentration of IgG to 0.4 mg/ml. After 30 minutes, the animals were transferred to 2 ml of cold hydra medium at 4°C (without DMSO or antibody) for 15 minutes and then allowed to slowly warm to room temperature.

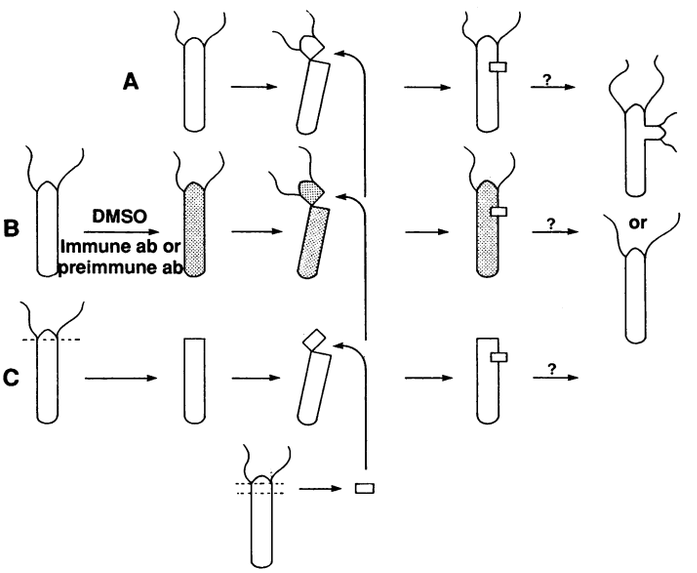


Fig. 7. Illustration of the experimental design. In all experiments, the apical quarter of the gastric region (the gastric region extends from the head to the first bud) of an untreated animal was laterally grafted to the midgastric region of (**A**) a normal, untreated animal, (**B**) an antibody-treated animal (shading represents the loaded preimmune or immune antibody), or a decapitated host (**C**). Each graft was scored 4 to 6 days later and placed into one of two classes: secondary axis formed, or no secondary axis formed. If the implant formed a clearly discernible head, with two to five tentacles surrounding a hypostome, it was considered to have formed a secondary axis. If the implant formed no recognizable structures, one to two small tentacles (with no hypostome), or a single large medial tentacle, it was considered not to have formed a secondary axis. Transplantation was carried out using the technique described previously (*31*). The ring of donor tissue (region 1) was threaded onto nylon fishing line (8-pound test). A slit was cut in the side of the host body column in the midgastric region at the 2/3 border and the fishing line was passed up through the slit and out through the mouth or decapitated end. The donor fragment was oriented so that its apical end faced away from the host animal. The donor and host tissue were pushed into contact and held together with short lengths of PE10 tubing (inner diameter slightly larger than the fishline) threaded over the ends of the fishing line. The completed grafts were allowed to heal for at least two hours before removal from the line. The results of the grafts were scored 4 to 6 days after the operation. The different classes of operations were interleaved on any given day of grafting to ensure that any variations in the animals or effects of time of day were represented.

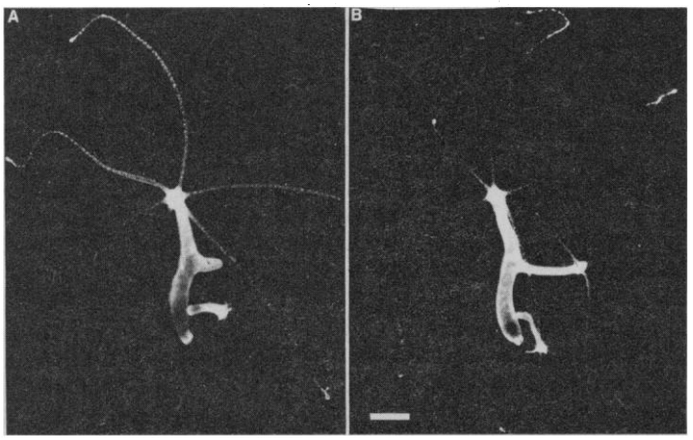


Fig. 8. The two types of experimental result obtained from the grafting operation described in Fig. 7. (**A**) An implant that did not form a secondary axis. The implant will eventually be resorbed into the body column. (**B**) An implant that formed a secondary axis. In both (A) and (B) the result of the implant is the outgrowth near the head; the secondary axis closer to the foot is a normally developing bud (scale bar, 1 mm).

possible that, since the antibody was applied primarily to the outside surface of the animal, the blockade of coupling in the endoderm was more short-lived and less complete than in the ectoderm. Finally, loading of the antibody into the hydra cells with DMSO does not uniformly fill all of the cells with antibody, as seen by treating the cells with FITC-antibodies. The presence of some cells that are less completely filled with the antibody might produce a partial return of gap junctional communication faster than expected from the dye passage experiments. Given these factors, it is not surprising that the antibody treatment is not as effective as decapitation in reducing head inhibition at the implant site.

Nonetheless our results indicate that cell-cell communication through gap junctions plays a role in the head inhibition process, most probably by providing a pathway for the movement of a diffusible substance from cell to cell. These results provide direct evidence consistent with the involvement of a diffusible substance moving through gap junctions in head inhibition in hydra. Previous work showed that the behavior of the head inhibition was consistent with a diffusible head inhibitor, but provided no direct evidence (*7*). Our results do not show that head inhibition is solely due to a single diffusible substance as proposed by most models of head inhibition (*11*, *12*), although they are consistent with this simple and attractive idea. Head inhibition could be a more complicated process involving, for example, the cooperative influences of both diffusible and nondiffusible components, or an electrically driven passage of ions or small molecules through the gap junctions. At present, too little is known of the full consequences of abolishing gap junctional communication in hydra to design or perform decisive test experiments of these intriguing alternatives.

Almost all previous attempts to determine the role of gap junctions in development have been largely limited to correlational studies that show the presence or absence of cell coupling at developmentally relevant locations (*5*). For example, dye coupling between the cells of the insect epidermis has been shown to be strong within each segment but restricted at the segment borders (*28*). The availability of specific immunological reagents for the major gap junction protein now permits new experimental approaches to this question. The first experiments of this class employed antibodies to the gap junction to disrupt cell-cell communication in the early amphibian embryo, and demonstrated a consistent set of developmental defects (*19*). These results indicate that some aspect of development was perturbed, but it remains for future work to identify precisely which processes were altered by the closure of the gap junctions.

Our experiments extend the previous work with antibodies against the gap junction by demonstrating the disruption of a defined developmental process, head inhibition in hydra. This not only lends further support to the proposal that gap junctions are important in development, but also provides evidence that gap junctions are involved in the patterning of tissues by permitting the exchange of diffusible substances between cells. The paradigm of loading the reagents into most or all of the cells of a tissue provides the means of testing the involvement of gap junctions in other multicellular systems. In hydra, this approach should permit the examination of several patterning processes and the determination of which of them are dependent on gap junction mediated communication.

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