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Functional Gap Junctions Are Not Required for Muscle Gene Activation by Induction in *Xenopus* Embryos

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Abstract. Muscle gene expression is known to be induced in animal pole cells of a *Xenopus* blastula after 2–3 h of close contact with vegetal pole cells. We tested whether this induction requires functional gap junctions between vegetal and animal portions of an animal–vegetal conjugate. Muscle gene transcription was assayed with a muscle-specific actin gene probe and the presence or absence of communication through gap junctions was determined electrophysiologically. Antibodies to gap junction protein were shown to block gap junction communication for the whole of the induction time, but did not prevent successful induction of muscle gene activation. The outcome was the same whether communication between inducing vegetal cells and responding animal cells was blocked by introducing antibodies into vegetal cells alone or into animal cells alone. We conclude that gap junctions are not required for this example of embryonic induction.

ANIMAL pole cells of the *Xenopus laevis* embryo do not normally express genes characteristic of mesodermal differentiation, such as muscle. However, these cells, which are destined to give rise to ectodermal derivatives in the course of normal development, can be induced to express the muscle-specific cardiac actin gene and to form morphologically and immunologically recognizable skeletal muscle after direct contact with blastula stage vegetal pole cells (Nieuwkoop, 1969; 1977; Gurdon et al., 1985; Dale et al., 1985). For the induction of muscle differentiation to be successful, animal pole cells must be close to cells of the vegetal pole for at least 2 h beginning at the blastula stage (Gurdon et al., 1985). Muscle gene expression is observed when animal cells have reached the equivalent of the midgastrula to early neurula stage, according to the sensitivity of the methods used (Cascio and Gurdon, 1986). This is the stage when muscle genes are activated in normal development (Mohun et al., 1984). Induction does not take place between dispersed animal and vegetal cells (Sargent et al., 1986) and appears to depend on contact, or at least close proximity (Gurdon et al., 1985).

Several soluble factors have been reported to have inductive effects. Some of these, for example lithium chloride, may have their effect because they bypass the primary inductive mechanism and activate some later step in the process. Such experiments do not, therefore, help in understanding the initial stages of normal induction.

The mechanism for the transfer of inductive signals has not yet been elucidated for any inductive interaction. In these circumstances it is important to separate out the various possible pathways through which such signals might travel. The elimination of potential pathways would be a considerable advance in our present state of knowledge since it could point the way for more precise experiments. The requirement for close contact between vegetal and animal pole cells immediately raises the possibility that this inducing signal may be transferred through a pathway such as that mediated by gap junctions. Gap junctions, which allow the direct transfer of ions and small molecules from cell to cell without recourse to the extracellular space, are known to link cells of the blastula stage *Xenopus* embryo (Palmer and Slack, 1970; Regen and Steinhardt, 1986).

The hypothesis that the inducing signal is transmitted from vegetal to animal pole cells through gap junctions can be directly tested using antibodies raised against the major 27-kD protein electrophoretically eluted from isolated rat liver gap junctions (Green, C. R., R. M. Earls, C. M. Jewell, K. Waymire-Purdue, L. L. Satterwhite, and N. B. Gilula, manuscript submitted for publication). These antibodies have been shown to block completely the direct communication between cells of the amphibian embryo (Warner et al., 1984). Furthermore, injection of gap junction antibodies into one cell of the animal pole at the 8 cell stage generates tadpoles with developmental defects that are consistent with the notion that the block of gap junctional communication interferes with neural induction (loc cit).

In this paper we test whether blastula stage vegetal pole cells remain able to induce muscle differentiation when all vegetal pole cells or all animal pole cells in a vegetal–animal conjugate have been rendered unable to communicate

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through gap junctions. This is one of the few cases where the function of gap junctions in development has been tested directly.

Materials and Methods

Embryos were obtained from mature *Xenopus laevis* induced to mate and lay eggs by injection of chorionic gonadotrophin (Pregnyl; Organon Ltd, Cambridge, United Kingdom). The embryos were staged according to the life table of Niuewkoop & Faber (1956). The eggs were stripped of their jelly coats by brief treatment with 2.0% cysteine in Holtfreter's solution (60 mM NaCl, 1 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM Tris-hydroxymethyl amino methane) at pH 8.0. On some occasions a single drop of 0.01% papain was added to the dejellying solution.

Injections were made into either all four vegetal cells or all four animal cells at the 8 cell stage. During injection the embryos were bathed in Holtfreter's solution at pH 7.4 containing 5% Ficoll 400 (Sigma Chemical Co., London) to allow easy orientation. After injection the embryos were left in Ficoll solution to aid wound healing. The Ficoll was then gradually diluted out with Holtfreter solution and the embryos then left in 20% Holtfreter solution until they reached the blastula stage. The embryos tolerated the injections well and continued to divide at the same rate as uninjected siblings.

Approximately 10 nl of one of two polyclonal antibodies raised against the major 27-kD protein electrophoretically eluted from isolated rat liver gap junctions at 1.5 mgm/ml was pressure injected into each cell. Before use the antibodies were twice affinity purified against the 27-kD protein (for details see Warner et al., 1984). As a control, preimmune IgGs affinity purified from the serum of the two rabbits used to raise the antibodies before immunization were injected.

The embryos were transferred to modified Barth's solution (Gurdon, 1977) or Holtfreter's solution at the blastula stage and removed from the vitelline membrane. The mesoderm was cut away and conjugates made of vegetal pole and animal pole tissue as described by Gurdon et al. (1985). Either the vegetal pole or the animal pole tissue came from embyros previously injected with gap junction antibody or preimmune IgGs. In each experiment some additional conjugates were constructed entirely from uninjected embryos.

Some conjugates were tested electrophysiologically for the presence or absence of electrical coupling when intact siblings had reached stage 10½ (~4 h after conjugation). On some occasions the animal cap was then removed. Either the animal cap alone, or the complete conjugate was cultured in modified Barth's solution until intact siblings reached Nieuwkoop and Faber stage 18. All samples were then frozen.

The abundance of cardiac actin and cytoskeletal actin mRNAs was determined as described in Gurdon et al. (1985). This involves the in vitro synthesis of highly radioactive antimessage RNA with SP6 RNA polymerase from a double-stranded cardiac actin cDNA attached to an SP6 promotor. The antimessage probe hybridizes to cardiac actin gene transcripts. RNAase was used to digest unhybridized probe and hence to represent quantitatively the abundance of actin gene transcripts. Details of the procedure are described by Melton et al. (1984). The strength of the induction was assessed from the ratio of cardiac to cytoskeletal actin mRNAs.

Microinjection

Glass micropipettes were pulled from thin-walled glass (Corning 7740; Glass Company of America; Bargaintown, NJ) and the tip of the pipette back-filled with the appropriate injection solution. The pipette was then attached to a Picospritzer II (General Valve Corporation, Fairfield, NJ) through a holder that allowed the application of pressure pulses to the back end of the pipette. The tip was broken back to a diameter of ~5 μm and the Picospritzer set to deliver volumes of 10 nl. The pipette was inserted into either vegetal or animal pole cells of the 8 cell stage embryo and antibody or preimmune IgGs injected by brief pressure pulses. The back pressure exerted by the cytoplasm was rather variable and the meniscus at the top of the column of fluid was always observed to ensure that fluid was leaving the pipette.

Electrophysiological Measurements

Electrical coupling within the conjugates was determined using standard electrophysiological techniques. One microelectrode (70–100 megohm resistance, filled with 0.8 M K citrate) was inserted into each of two cells about four cells apart. One electrode was used to inject rectangular depolarizing or hyperpolarizing current pulses (1-s long, up to 150 nA) while the second recorded the membrane potential and the resultant electrotonic potential. The signals were displayed on an oscilloscope (5000 series; Tektronix, Inc., Beaverton, OR) and a channel pen recorder (brush 4; Gould, Inc., Cleveland, OH). The electrodes made contact with the input of high impedance amplifiers through Ag/AgCl half cells and the bath was earthed through similar half cells. The current was recorded across a 100-kohm resistor in the earth return circuit. Both electrodes were inserted in the voltage recording mode and the appearance of the membrane potential used to indicate the intracellular location of each electrode; one electrode was then switched over to current injection.

The appearance of an electrotonic potential on the second electrode indicated the presence of electrical coupling. When either electrode was moved into the intercellular space the electrotonic potential disappeared, confirming that current flow from cell to cell was not taking place through the intercellular spaces. This method does not allow quantitative estimation of the strength of electrical coupling because the properties of both the surface and junctional membranes determine how much current flows through the gap junction. However, provided the interelectrode spacing is kept fairly constant from experiment to experiment, a qualitative indication of the relative efficiency of electrical coupling can be obtained. Electrical coupling was concluded to be absent when a current pulse of 150 nA produced no detectable deflection on the voltage trace. An electrotonic potential could be recognized unequivocally, provided the voltage deflection produced by injection of current was >0.3 mV. Electrical coupling was expressed as a transfer resistance (mV/10 nA); the limit of detection given above is equivalent to a transfer resistance of 0.02 mV/10 nA.

Results

Experimental Design

The basic design of the experiments is illustrated in Fig. 1. Detailed information is provided in Materials and Methods. At the 8 cell stage one of the two polyclonal antibody preparations used previously (Warner et al., 1984) was injected into all four vegetal pole cells or into all four animal pole cells. Preimmune antibodies (IgGs) were injected into other 8 cell stage embryos as a control. At the blastula stage the mesoderm was cut away and conjugates made of vegetal and animal pole tissue. Either the vegetal or the animal pole tis-

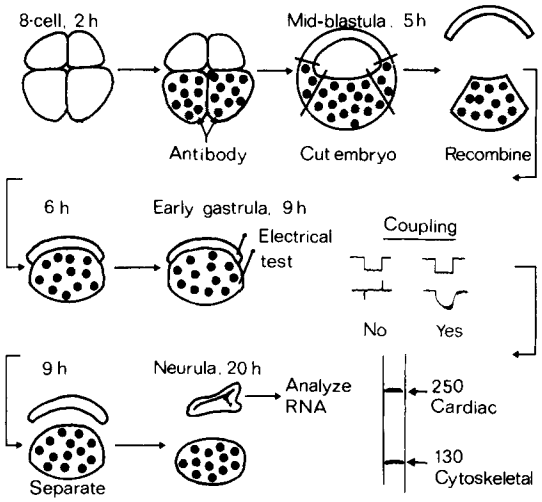

Figure 1. Diagram of experimental design. In the example shown, antibodies were injected into the four vegetal cells of an 8 cell stage embryo (shown from side view), and antibody-containing vegetal tissue subsequently combined with animal tissue from an uninjected embryo. Other experiments were conducted in the converse way, antibodies being injected into the four animal cells of an 8 cell stage embryo.

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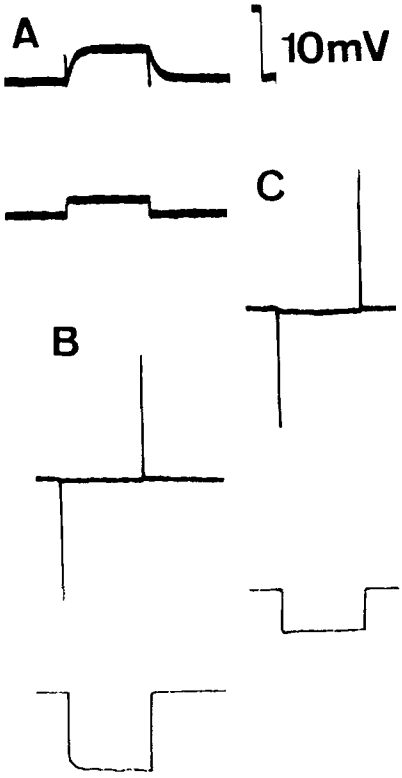

Figure 2. Electrical coupling recorded in conjugates across the animal-vegetal margin with about four cells interposed between current-injecting and voltage-recording electrodes. (*A*) Coupling in a control conjugate 40 min after construction; (*lower trace*) 4 nA, 1-s long, depolarizing current pulse injected into a vegetal pole cell, membrane potential, −55 mV; (*upper trace*) resultant electrotonic potential recorded in the animal pole, membrane potential −63 mV, transfer resistance 10 mV/10 nA. (*B*) Absence of coupling in a conjugate made with vegetal pole cells containing gap junction antibody measured 3½ h after construction; (*lower trace*) 50 nA, 1-s hyperpolarising current pulse injected into a vegetal pole cell (note gain is half that in *A*), membrane potential −30 mV; (*upper trace*) record of voltage response in an animal pole cell, membrane potential −26 mV. Note absence of electrotonic potential, with capacitative artefact remaining on the trace. (*C*) Weak coupling in a conjugate with vegetal pole cells containing gap junction antibody 4 h after conjugation; (*lower trace*) 22.5 nA, 1-s hyperpolarizing current pulse injected into vegetal pole cell, membrane potential −35 mV; (*upper trace*) electrotonic potential recorded in animal pole cell (0.5 mV), membrane potential −30 mV, transfer resistance 0.2 mV/10 nA. Records deliberately chosen to illustrate just detectable voltage response. Rise time of the electrotonic potential obscured by capacitative artefact.

sue came from embryos previously injected with gap junction antibody or with preimmune IgGs. Additional conjugates were constructed from uninjected siblings.

When intact siblings reached stage 10½ (~4 h after conjugation) electrophysiological tests were made for the presence or absence of electrical coupling. In some cases the animal cap was then removed and either the animal cap alone, or the complete conjugate, was cultured in modified Barth's solution until intact siblings reached stage 18. All samples were then frozen and assayed for the presence of cardiac actin mRNAs (specific for axial skeletal muscle and indicating induction), and also for cytoskeletal actin mRNAs (which are synthesized in the absence of induction and which provide a correction for the amount of cellular material in conjugates of different sizes), as described in Gurdon et al. (1985). The strength of the induction was assessed from the ratio of cardiac to cytoskeletal actin mRNAs.

Gap Junctions Are Formed Rapidly in Control Conjugates

A role for gap junctions in the inductive interaction between vegetal and animal pole cells is only plausible if gap junctions are established between these cells relatively soon after conjugation, within the minimum period of two hours necessary for the inductive interaction. Electrical coupling between vegetal and animal pole cells was determined in seven control conjugates at intervals ranging from 40 to 100 min after reconjugation. The cells had resting potentials between −20 and −60 mV, which is within the normal range for *Xenopus* embryonic cells at these stages (Slack and Warner, 1975; Warner, A. E., unpublished observations). In all cases good electrical coupling was observed. An example of an electrotonic potential recorded 40 min after reconjugation in an animal pole cell as a result of injecting a small, depolarizing current pulse into a vegetal pole cell is shown in Fig. 2 *A*. All the results are given in Table I. The transfer resistances ranged from 2.2 to 14.5 mV/10 nA, at least 100 times greater than the limit of detection. The variability probably arises both from differences in surface membrane resistance from embryo to embryo and from occasional errors in estimating the number of cells interposed between current-injecting and voltage-recording electrodes. No clear difference was observed between conjugates made 40 or 100 min before the measurements were taken. Thus, communication through gap junctions is established between vegetal and animal pole cells well within the minimum time for the inductive interaction estimated by Gurdon et al. (1985).

Suppression of Communication through Gap Junctions in Inducing Vegetal Cells

Conjugates constructed from embryos where the vegetal or the animal portion had previously been injected with either of the gap junction antibodies healed well and showed no tendency to separate if disturbed. In all cases the membrane

Table I. Electrical Coupling between Animal and Vegetal Pole Cells in Control Recombinants at Different Times after Recombination

Recombinant Number	Time after recombination	Transfer resistance
	min	*mV/10 nA*
1	40	14.5
2	40	3.8
3	55	8.0
4	60	3.0
	90	2.15
5	65	13.3
6	90	9.3
7	100	2.2

Measurements were made across the animal-vegetal margin in each recombinant with about four cells separating current-injecting and voltage-recording microelectrodes. Values given for the transfer resistance are the average of at least two determinations at different positions within each recombinant.

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Table II. Electrical Coupling between Vegetal and Animal Pole Cells in Recombinants with Gap Junction Antibody in the Vegetal Pole Cells

Number of Recombinants	Transfer resistance	Coupling
	mV/10 nA	
Gap junction Antibody		
19	Not detectable	Uncoupled
3	0.04, 0.16, 0.13	Weakly coupled
1	2.0	Coupled within normal range (see Table I)
Preimmune IgGs		
11	Mean 0.42, range 0.12–0.8	Less well coupled than controls

Measurements were made across the animal–vegetal margin of each recombinant with about four cells separating current-injecting and voltage-recording electrodes. Values for transfer resistance are the average of at least two measurements at different positions in each recombinant. Limit of detection: electrotonic potential of <0.3 mV for 150 nA current pulse, equivalent to 0.02 mV/10 nA.

potentials were within the normal range of −20 to −60 mV, confirming that the gap junction antibody has no effect on the resting membrane potential (cf. Warner et al., 1984).

Fig. 2 *B* shows coupling measurements between antibody-injected vegetal cells and uninjected animal portions in one of these conjugates, made 4 h after vegetal and animal pole cells had been placed into contact. Resting potentials of −20 and −24 mV were recorded in animal and vegetal cells. A 50-nA hyperpolarizing current pulse injected into a vegetal pole cell did not produce a detectable electrotonic potential in an animal pole cell; vegetal and animal pole cells had failed to establish electrical coupling. Fig. 2 *C* shows an example of a conjugate where current injected into a vegetal pole cell produced a just detectable electrotonic potential in an animal pole cell, indicating that weak communication through gap junctions was present between vegetal and animal cells.

23 conjugates from four independent experiments where the vegetal pole cells contained one of the gap junction antibodies were examined between 3½ and 4½ h after conjugation. Assessment of the absence of electrical coupling was based on four or five measurements made at different points around the animal–vegetal margin, each of which failed to reveal electrical coupling between animal and vegetal cells. The starting point for these measurements was taken at random. Even one measurement showing detectable electrical coupling automatically put the conjugate into the coupled class. Extensive testing was rarely necessary to demonstrate the presence of electrical coupling between vegetal and animal cells. Consequently, each assessment of the presence of electrical coupling is based on at least two measurements in different regions of the conjugate.

Table II shows that on the criteria given above, 19 (83%) showed no detectable electrical coupling. In the four other conjugates, one was as well coupled as the controls (transfer resistance 2.0 mV/10 nA), two gave transfer resistances of 0.13 and 0.16 mV/10 nA, and one a transfer resistance of 0.04 mV/10 nA. Both antibody preparations were equally effective. 10 conjugates from embryos injected with preimmune IgGs were all electrically coupled, with transfer resistances between 0.12 and 0.8 mV/10 nA (Table II). These results indicate that the injection of gap junction antibody into the vegetal pole can prevent vegetal and animal portions from establishing functional communication through gap junctions at least until stage 10½. The reason for the reduced electrical coupling in conjugates made from embryos injected with preimmune IgGs is not clear. Previous tests of dye transfer between animal pole cells did not suggest any inhibition of communication by preimmune antibodies (Warner et al., 1984). The longer interval between injection and measurement in the present experiments may reveal some low level, nonspecific effect of these mixed antibodies, which had not been affinity purified against identified antigens.

Fig. 3 illustrates the analysis of muscle and cytoskeletal actin mRNAs in one of our experiments. The RNAase protection procedure, using SP6 generated probes, is summarized in Materials and Methods. We compared muscle gene activa-

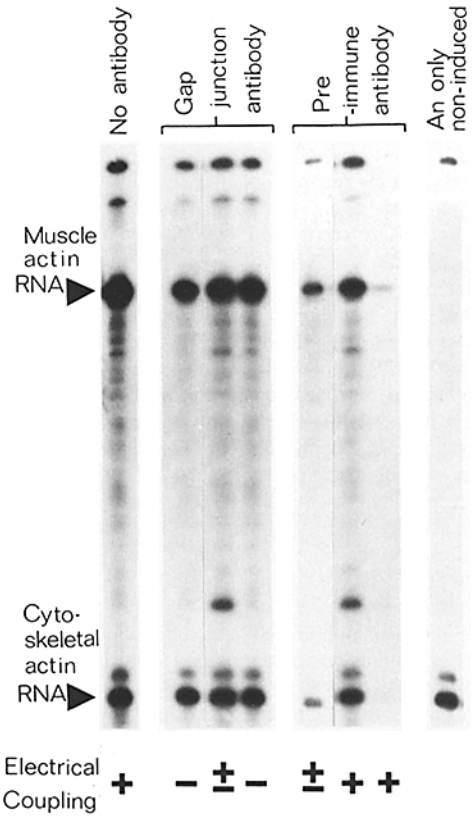

Figure 3. Nuclease protection analysis of actin gene transcripts in conjugate embryos, having inhibited gap junctions in their vegetal cells. Anti-gap junction protein antibodies or preimmune IgGs were injected into vegetal cells, and conjugates made, as illustrated in Fig. 1. Electrical coupling (+, coupled; −, uncoupled) between the animal and vegetal cells was tested on each embryo at the equivalent of the early gastrula stage. Conjugate embryos were frozen when controls had reached stage 18. RNA was estimated and analyzed with an SP6 probe as described by Gurdon et al. (1985). Each track shows the analysis of half of one conjugate embryo. The An only sample is one in which two animal pieces were placed in contact (in the absence of any inducing vegetal cells). The radioactive RNA probe, which is initially about 380 nucleotides long, is protected for 285 nucleotides by cardiac actin RNA, and for about 135 nucleotides by cytoskeletal RNA.

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tion in uncoupled, antibody-containing conjugates with activation in coupled conjugates containing preimmune IgGs. An uninjected control was also included. The main result was that muscle actin RNAs were present in similar amounts in all cases. The top part of Table VI summarizes the results of SP6 analyses for all the experiments in this series, together with information from Table II on the presence or absence of electrical coupling between animal and vegetal pole cells. The effectiveness of induction by the vegetal pole was assessed by the ratio between muscle actin RNAs and cytoskeletal actin RNAs averaged for each group. Animal caps removed from vegetal pole portions at stage 10½ and complete conjugates gave identical results and are therefore not individually identified. Some conjugates made from injected embryos activated muscle actin RNAs at a lower level than the controls, giving values below 100%. However, there was no difference in this respect between conjugates designated as coupled or uncoupled, or between conjugates made from embryos injected with gap junction antibody and those injected with preimmune IgGs. The overall conclusion from these experiments is that it is not necessary for vegetal pole cells to establish functional communication through gap junctions with animal pole cells for vegetal pole cells to exert their normal inductive effect.

The Consequences of Reducing the Number of Inducing Vegetal Pole Cells

It is important to know how many vegetal cells are required to induce muscle gene activation to the usual extent. For example, if 10% of vegetal cells could give a maximal induction, and this induction required functional communication through gap junctions formed between vegetal and animal pole cells, then even if coupling between 90% of cells remained blocked by gap junction antibody, no reduction in muscle gene activation would be observed. To determine the minimum number of vegetal cells able to induce a response in animal pole cells, we measured actin gene activation in normal conjugates made with progressively smaller vegetal pieces. Cells from the dorsal side of the vegetal pole were always included in order to maximize the chances of obtaining induction. The results of three experiments are given in Table III. When the vegetal piece was reduced to one-half, activation of cardiac actin genes fell to two-thirds of the control level, close to that observed in both coupled and uncoupled conjugates made with antibody-containing vegetal pole cells. However, reducing the vegetal piece to one-quarter of its normal size produced a steep reduction, with cardiac actin gene activation falling to <10% of the normal response. Thus, the strength of the inductive signal is critically dependent on the number of vegetal cells available to induce. These findings indicate that, to account for the levels of cardiac gene activation seen in antibody-injected conjugates, 50% or more of the cells in the vegetal pole would have to be available to form functional gap junctions.

Table III. A Reduction in the Number of Inducing Vegetal Cells Greatly Decreases the Level of Muscle Gene Activation

Fraction of vegetal piece in conjugate	Number of conjugates	Cardiac/cytoskeletal actin RNA ratio
		%
Whole	3	100
Half	3	68
Quarter	3	8

Conjugates were prepared with a piece of animal blastula tissue placed in contact with a whole, half, or quarter vegetal piece of a blastula. The half and quarter vegetal pieces were intended to include the dorsal region (opposite the sperm entry point, which had been marked with blue dye). Conjugates were incubated until stage 20–24, when they were frozen, extracted, and analyzed with the same cardiac actin probe as described in Materials and Methods. The cardiac/cytoskeletal actin ratio was determined by densitometry of gels of protected SP6 probe. For whole conjugates the ratio was 3.1 for this series.

Table IV. Electrical Coupling between Cells in the Animal Portion 4 h after Combination with Vegetal Pole Cells (No Antibody)

Recombinant Number	Transfer Resistance
	mV/10 nA
1	3.6
2	2.4
3	1.4
4	2.4
5	1.0
6	8.0
7	2.1
8	2.1
9	3.0
10	1.0

Measurements were made with about four cells interposed between current-injecting and voltage-recording electrodes. Transfer resistances based on average of at least two measurements in different regions of the animal pole.

Suppression of Gap Junctional Communication in Responding Animal Pole Cells

The finding that gap junction communication is not required for vegetal cells to induce animal cells does not preclude an essential role for gap junctions in the process by which animal pole cells respond to induction. We have tested this possibility using the experimental design of Fig. 1, except that gap junction antibody was injected into the four animal, rather than the four vegetal cells at the 8 cell stage. Because we were specifically testing for a requirement for gap junctional communication between animal pole cells, in this series the effectiveness of the antibody was assessed by measuring electrical coupling between cells in the animal portion of the conjugates when sibling intact embryos had reached stage 10½. Table IV gives transfer resistances determined with the electrodes about four cells apart in the animal pole of 10 conjugates from uninjected embryos from three independent experiments. The values range from 1 to 8 mV/10 nA, and are about the same as those recorded with a similar interelectrode spacing across the animal–vegetal margin (Table I).

15 conjugates made from embryos injected with a gap junction antibody into the animal pole showed a spectrum of transfer resistances (Table V), as also observed in embryos where the vegetal pole had been injected (Table II). The same criteria apply to these measurements as for measurements in the vegetal pole detailed above. Five (33%) showed no detectable electrical coupling, indicating that gap junction antibody injected at the 8 cell stage was probably still fully effective. Five (33%) proved to be electrically coupled, but with

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Table V. Electrical Coupling between Cells in the Animal Part of Recombinants Made with Animal Cells Containing Gap Junction Antibody

Number of recombinants	Transfer resistance	Coupling
	mV/10 nA	
5	Not detectable	Uncoupled
5	Mean 0.18, range 0.04–0.5	Weakly coupled
5	Mean 1.1, range 0.7–1.9	Close to control (Table IV)

Measurements were made with about four cells between current-injecting and voltage-recording electrodes in the animal pole. Values for transfer resistance based on at least two measurements in different regions. Limit of detection, electrotonic potential of <0.3 mV for 150 nA current pulse, equivalent to 0.02 mV/10 nA.

transfer resistances between 10 and 100 times lower than observed in controls. The remainder were almost as well coupled as uninjected cells from the animal pole, with transfer resistances between 0.7 and 1.9 mV/10 nA. Cells in the animal pole seem to escape from the block of gap junctional communication imposed by the antibody rather sooner than cells in the vegetal pole. This may be related to the more rapid division of animal pole cells once past the midblastula transition. Consequently, conclusions about the importance of gap junctional communication for muscle gene activation in responding animal cells may only be valid up to stage 10½, which is close to the time when animal portions lose their responsiveness to induction by the vegetal pole (Gurdon et al., 1985).

Fig. 4 shows SP6 analyses of muscle and cytoskeletal actin mRNAs from one experiment where the animal portion of some conjugates contained gap junction antibody. Both noninjected and antibody-containing animal portions show some variability in the amount of muscle actin RNAs, but this was not related to the presence, absence, or effectiveness of the gap junction antibody. The results of three independent experiments are summarized in the bottom half of Table VI and

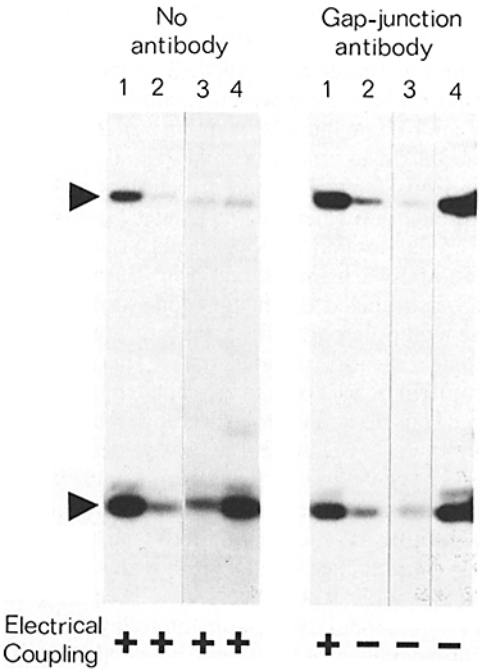

Figure 4. Nuclease protection analysis of actin gene transcripts in conjugate embryos having inhibited gap junctions in their animal cells. The first three lanes show SP6 analyses of uninjected, control conjugates. Animal pole cells from this batch happened to activate muscle genes at a lower level than is usual; in other respects, see legend to Fig. 3.

confirm that activation of muscle-specific actin mRNAs is independent of the presence or absence of electrical coupling within the responding animal pole cells during the inductive interaction.

These two sets of experiments suggest that the ability of vegetal pole cells to induce muscle gene activation in the animal pole is unrelated to the efficiency of electrical coupling between the inducing and the responding cells, or to electrical coupling within the animal regions.

Table VI. Quantitative Analysis of Muscle Gene Transcription in Animal–Vegetal Conjugates with Inhibited Gap Junction Communication

Type of conjugate	Antibody injection	Electrical coupling	Number of assays	Cardiac as percent of cytoskeletal actin mRNAs*
Antibody in vegetal cells				
An:Veg	Anti-gap junction	+	4	46
An:Veg	Anti-gap junction	−	11	67
An:Veg	Preimmune IgGs	+	7	61
An:Veg	None	+	10	96
An:An	None	Not tested	10	0
Antibody in animal cells				
An:Veg	Anti-gap junction	+	2	90
An:Veg	Anti-gap junction	−	4	81
An:Veg	Anti-gap junction	Not tested	3	108
An:Veg	None	+	4	41

+ Conjugates with electrical coupling above 0.02 mV/10 nA (the limit of detection).
− Conjugates with electrical coupling below 0.02 mV/10 nA.
* Values obtained by densitometry of appropriately exposed gels of SP6 analyses.
Each part of the table gives results pooled from three separate experiments.

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Discussion

When vegetal pole cells of the blastula stage amphibian embryo are placed into direct contact with animal pole cells, gap junctions between the two populations are rapidly established. However, it seems unlikely that the pathway for cell communication mediated by gap junctions plays a part in the transmission of the inductive signal leading to the activation of genes characteristic of axial muscle. Antibodies that specifically prevent communication through gap junctions have no adverse effect on induction, whether communication is blocked in the inducing vegetal cells or within the responding tissue during the time when vegetal pole cells exert their inductive effect. Because this conclusion is based on functional measurement of direct cell to cell communication, it does not depend on the mechanism by which these antibodies achieve the block.

Electrophysiological measurements of coupling between animal and vegetal portions, or within the animal portion, of these relatively large conjugates ($\sim 10^4$ cells at the end of the inductive period) would not have revealed a small number of functional gap junctions between cells that had escaped from the communication block imposed by the antibody. An unequivocal answer to the question whether all cells in the vegetal pole remain unable to establish functional coupling with animal pole cells can only be achieved by testing coupling between every animal–vegetal pair. This is simply not practicable. If we assume that vegetal cells randomly escape from the block of communication imposed by the antibody, the likelihood of obtaining five successive measurements indicating the absence of electrical coupling between vegetal and animal cells can be estimated using the binomial theorem. On this basis the likelihood that 55% of cells have escaped is 0.09%. The likelihood that 25% of cells are coupled is 10%. It is, in fact, unlikely that cells escape the block entirely at random, since cells in each quadrant of the vegetal pole are the progeny of one of the four cells injected at the 8 cell stage. The antibody diffuses rapidly throughout the cell at the 8 cell stage (see Warner et al., 1984), so that the progeny will contain equivalent amounts of antibody. Other experiments have shown that cells containing equivalent amounts of antibody and dividing at a similar rate (as is the case for vegetal pole cells) tend to escape the block simultaneously. A worst case estimate can be made by supposing that all the conjugates are identical and that the pooled results (see Table II) reflect the incidence of coupled and uncoupled cells within each conjugate. This would suggest that $\sim 20\%$ of cells remain coupled despite the presence of the gap junction antibody.

We can, therefore, be reasonably certain that 75–80% of antibody-containing cells are still unable to communicate at the end of the inductive period. When only 25% of vegetal pole cells are available to induce, the response in the animal pole declines to less than one-tenth of its normal level. Thus, if gap junctions are important in transmitting the signal from vegetal to animal cells, even if 25% of vegetal pole cells had escaped from the effect of the antibody, there should have been a 10-fold reduction in activation of cardiac actin genes. Conjugates designated as uncoupled nevertheless activated cardiac actin genes at $\sim 70\%$ of the control level, with no difference between coupled and uncoupled conjugates (Table VI).

The overall conclusion from these arguments is that it is unlikely that the inductive signal emitted by vegetal pole cells leading to induction of muscle differentiation in animal pole cells is transmitted through gap junctions. These experiments provide one of the few cases in which the function of gap junctions in development has been rigorously tested and they define more closely than hitherto the possible mechanisms by which muscle gene activation is induced. Our results imply that this signal is transferred through some mechanism other than gap junctions, which nevertheless requires close proximity of vegetal and animal pole cells (Gurdon et al., 1985; Sargent et al., 1986). The elimination of the gap junctional pathway should allow the design of experiments that are more precisely directed towards understanding the mechanism of this inductive interaction. Possibilities include interaction through molecules of the extracellular matrix or the release into the intercellular space of a molecule that is rapidly destroyed once it leaves cells of the vegetal pole. Either of these mechanisms would meet the requirement for close contact, but would probably not require the generation of an intercellular membrane structure in order to mediate the interaction. At present, further conclusions about the mechanism underlying this particular inductive interaction cannot be drawn. The failure of vegetal pole and animal pole cells to establish electrical coupling when gap junction antibody is present in vegetal cells does not imply that the antibody inhibits the formation of gap junctions; gap junctions might be formed but might be nonfunctional. This issue can only be resolved by ultrastructural examination of completely uncoupled conjugates.

Our experiments show that the block of gap junctional communication in the animal pole is maintained until stage 10½, so that communication in responding animal cells is not necessary while animal cells are competent to respond to induction by vegetal cells (Gurdon et al., 1985). However, it is possible that gap junctional communication between animal cells may be required after stage 10½ for normal gene activation, since gap junction antibodies injected into the animal pole are beginning to lose their effectiveness by this stage.

Previously it was found that the progeny of the right hand dorsal animal pole blastomere injected with either of the gap junction antibodies at the 8 cell stage failed to communicate at the 32 cell stage (Warner et al., 1984). The present experiments demonstrate that the block of direct cell to cell communication initiated by the injection of antibodies to gap junction protein at the 8 cell stage can last till at least stage 10½ (6–7 h), the latest time point tested, when neural induction is just beginning. 66% of animal portions were still completely or partially communication defective. This is strikingly similar to the proportion of tadpoles (63%) found by Warner et al. (1984) to develop patterning defects in the region derived from the antibody injected blastomere. We conclude that the absence of a need for gap junction communication during mesoderm induction does not exclude a role for gap junctions in neural induction.

In the course of normal development, mesoderm cells not only differentiate into the appropriate cell types, but also are organized to form the orderly structures of the embryonic axis. The mechanism whereby mesoderm cells are patterned into these structures is not addressed in our experiments, which use the appearance of cardiac actin mRNAs as the criterion for successful mesodermal induction. Gap junc-

tions may be involved in this patterning process, as suggested by preliminary experiments in which gap junction antibody was injected into vegetal pole cells of *Xenopus laevis* embryos and the embryos raised to stage 36/37 (swimming tadpole). These embryos develop with deficiencies in axial structures, and in some cases with no axis at all, although mesoderm cells can always be identified within the disorganized embryo (Warner, A. E., S. C. Guthrie, and N. B. Gilula, unpublished, quoted in Warner, 1985; 1986). Taken together with the results reported in this paper, these findings suggest that the generation and organization of mesoderm cells may involve two inductive mechanisms, one that induces gene activation and is not transferred through gap junctions and a second that requires gap junctional communication in order to pattern the structures formed by mesodermal cells.

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