J. Embryol. exp. Morph. **89, Supplement,** *365–380 (1985)*
Printed in Great Britain © *The Company of Biologists Limited 1985*

The role of gap junctions in amphibian development

ANNE E. WARNER

Department of Anatomy and Embryology, University College, London, Gower Street, London WC1E 6BT, U.K.

SUMMARY

The possibility that communication through gap junctions may be important during embryonic development has often been raised since gap junctions were first described between early embryonic cells. It is now known that this direct cell-to-cell communication pathway disappears between groups of embryonic cells with different developmental fates as the embryo progresses through development, suggesting that transfer through the gap junctional pathway may play some part in controlling events during development. Supportive evidence for a role for gap junctions comes from experiments demonstrating that the properties of gap junctions differ at the border separating each segment in insect epidermis. Recently it has been shown that the ability to exchange small dyes between cells in the amphibian embryo depends on the position of each cell with respect to the grey crescent. When communication through gap junctions is prevented, by injecting antibodies to gap junctions protein, pattern formation is severely disturbed in the non-communicating region. The paper describes experiments on the pattern of junctional communication at early stages of development of the amphibian embryo and illustrates how anti-gap junction antibodies are being used to determine when and where communication through gap junctions may play an important role during development.

INTRODUCTION

Cells of the early amphibian embryo, like those in all other species so far examined, are able to communicate with each other through a direct cell-to-cell communication pathway (Ito & Lowenstein, 1966; Palmer & Slack, 1970), which is probably mediated by the intercellular structure called the gap junction. At early stages all cells in the embryo communicate through this channel and this has led many authors to speculate that cellular interactions occuring during development may be mediated by the transfer of instructive molecules through gap junctions (e.g. Potter, Furshpan & Lennox, 1966). Despite the attraction of this hypothesis it has proved remarkably difficult to provide unequivocal evidence for it. Previous approaches to this problem have focused on the timing of the elimination of gap junctions during early development, to see whether the communicating channel either disappears between groups of cells as they diverge along different developmental pathways or is formed between cells when they begin to interact. Gap junctional communication is normally detected electrophysiologically when low electrical resistance pathways between cells reflect the ability to exchange small

Key words: gap junctions, electrophysiological coupling, Lucifer Yellow, Texas Red, gap junction protein – antibody to, mesoderm induction, neural induction, amphibia.

ions. The transfer of larger molecules is examined by injection of fluorescent dyes, such as Lucifer Yellow, which do not cross the cell membrane. Alternatively gap junctions have been identified with the electron microscope. Since this Symposium is concerned with amphibian development I have chosen to quote experiments on the amphibian embryo, although similar results have been reported on a number of other systems.

Thus it has been shown that cells of the neural plate (destined to form the nervous system) and lateral ectoderm lose low electrical resistance connections as the neural tube closes, with new pathways being formed between ectoderm cells originally separated by the neural plate as they fuse in the mid-line (Warner, 1973). In the retina gap junctions disappear between retinal cells at about the time of specification of polarity (Dixon & Cronley-Dillon, 1972; 1974). In the mesoderm, myotomal mesoderm and overlying dermatome in *Xenopus laevis* have lost their gap junctions (detected electrophysiologically) before segmentation of the myotomes begins (Blackshaw & Warner, 1976). In the axolotl, where the dermatome segments along with the myotome, the dermatome and myotome remain able to communicate with each other after segmentation (Blackshaw & Warner, 1976). During segmentation itself the ability to communicate is lost between the unsegmented mesoderm and cells destined to form the next somite shortly before segmentation. In species where the segmented cells form a rosette containing both dermatome and myotome cells (e.g. the axolotl) each somite then remains isolated from its neighbours until the myotomes are formed (Blackshaw & Warner, 1976) when gap junctions become established between myotomal muscle cells while they are being innervated (Keeter, Pappas & Model, 1977). In *Xenopus laevis*, where the myotomes are formed immediately on segmentation and innervation follows shortly afterwards, gap junctions are established again as soon as a new myotome is generated (Blackshaw & Warner, 1976). Thus in both amphibian species gap junctions are present between myotomal muscle cells during the initial stages of formation of the neuromuscular junction. The possible functional role of direct cell-to-cell communication during innervation is not known, but junctions between the muscle cells disappear once the tadpole begins sustained swimming. The elimination of these gap junctions seems to be controlled by activation of the acetylcholine receptor (Armstrong, Turin & Warner, 1983).

Searches for the formation of gap junctions during inductive interactions have so far been few. Blackshaw (quoted by Warner, 1979) found that mesoderm and ectoderm layers of *Xenopus laevis* embryos are electrically coupled during gastrulation, while Warner (1973) and Sheridan (1968) noted that notochord and neural plate cells were in electrical communication during the neural plate stages. Rotated portions of the early neural plate are known to regulate and develop according to their new position (Jacobson, 1964). The time course of formation of electrical coupling between such grafts and host in the axolotl early neural plate was examined by J. M. W. Slack & A. E. Warner (quoted in Warner, 1981) who found that it took between 40 minutes and 3 hours for junctional communication to be

established. These authors also found that recombinants of dorsal and ventral mesoderm, between which inductive interactions occur, took a similar time to establish electrical coupling.

Such experiments are consistent with a role for gap junctional communication in the interactions which take place between embryonic cells but the precise relationship between the time of the elimination of gap junctional communication and the end of instructive interactions is not sufficiently well defined to make such a role compelling. It may be that a very precise correlation is not essential, as long as direct cell-to-cell communication ceases to be possible shortly after the end of an interactive event. If this is the case then control could be exerted at the level of gap junction protein synthesis rather than shutting down a once permeable channel.

Gap junctions have been shown to allow the transfer from cell to cell of molecules larger than small ions, with the limit lying in the region of molecules of about 1000 molecular weight (Simpson, Rose & Loewenstein, 1977). Although small ions seem to move without restriction from one cell to the next throughout the early embryo it is possible that differences in the ability to exchange molecules larger than small ions, but below the cut-off limit, may exist within the embryo. This is an attractive possibility for two reasons. Firstly it would allow cells to recognize the presence of a near neighbour without necessarily transferring all molecules below a certain size between them. Secondly it is already known that such variations in transfer can occur. In the epidermis of segmented insects such as *Oncopeltus* and *Calliphora* it has been found that cells which lie on either side of the segment boundary (known to mark the end of a developmental field – Locke, 1959; Lawrence, 1973) are electrically coupled across the segment boundary (Warner & Lawrence, 1973) but the ability to exchange small dyes in the 400–500 molecular weight range can be extremely restricted (Warner & Lawrence, 1982; Blennerhassett & Caveney, 1984). By contrast, cells within the same segment exchange Lucifer Yellow with facility. Since the segment boundary is known to be the site of a developmental boundary, these results re-inforce the view that communication through gap junctions may play a role in controlling developmental interactions. Such experiments also suggest that it is worth re-examining the patterns of dye transfer in embryos such as the amphibian embryo, where developmental boundaries cannot be identified by morphological markers, but may nevertheless be present.

DYE TRANSFER EXPERIMENTS IN *XENOPUS*

Guthrie (1984) examined transfer of the dye Lucifer Yellow (M.W. 450) between identified cells of the 32-cell-stage *Xenopus laevis* embryo using the primary cleavage axis and the grey crescent as markers for pigmented cells in the animal pole destined to form dorsal structures (in the region of the grey crescent) and ventral structures (opposite the grey crescent). She found a very high correlation between the ability to transfer dye and cell position. Cells lying in the future dorsal region transferred dye to their neighbours in 70 % of trials, while cells lying in future

ventral regions transferred dye in only 15 % of trials. Such a pattern is very unlikely to have arisen by chance ($P < 0{\cdot}001$, Chi-squared test). Positional dependence of the ability to exchange dyes through gap junctions is not restricted to the 32-cell stage. A similar pattern is present at the 16-cell and 64-cell stages of development (Guthrie, 1984). More recent experiments (Guthrie, Turin & Warner, manuscript in preparation) have revealed that when the embryo enters the 128-cell stage of development the ability of dorsal animal pole cells to transfer Lucifer Yellow to their neighbours declines to about 15 %, closely similar to that in ventral regions. Comparisons between cells lying in the animal (pigmented) and vegetal (unpigmented) hemispheres of the embryo show that up to the 128-cell stage, transfer of Lucifer Yellow between vegetal pole cells, whether dorsal or ventral, is a relatively rare event. Once the embryo enters the early blastula stage (256 cells) cells in the dorsal equatorial region (both pigmented and non-pigmented) once again frequently transfer (65 % of trials) Lucifer Yellow to their neighbours, although ventral equatorial cells are still relatively poor at exchanging dye with their neighbours.

The underlying developmental reason for these rather complex and changing patterns of junctional communication is not known. They cannot reflect the variable persistence of cytoplasmic bridges between the relatively rapidly cleaving cells since transfer of FITC-labelled dextrans, too large to move through gap junctions, is restricted to the sister cell from the previous cleavage only (Guthrie, 1984). Nevertheless such findings, like those in insect epidermis, lend support to the hypothesis that direct cell-to-cell communication through gap junctions is involved in cellular interactions during development.

INHIBITION OF GAP JUNCTION FUNCTION BY INJECTED ANTIBODY

Clearly the ability of cells to communicate through gap junctions changes in a number of rather complex ways during the development of the amphibian embryo and a real test of a role for gap junctions can only come from experiments in which junctional communication is suppressed completely and the developmental consequences can be observed. With the generation of antibodies directed against gap junctional proteins such experiments have, for the first time, become possible (Warner, Guthrie & Gilula, 1984). For a full description of these experiments the reader should refer to the original paper. Polyclonal antibodies were raised against the major, 27kD protein electrophoretically eluted from preparations of isolated rat liver gap junctions and then twice affinity purified against the 27kD protein. After injection into a dorsal, right-hand blastomere at the 8-cell stage (Fig. 1). The antibodies inhibit both dye transfer and electrical coupling assessed between cells in the dorsal, animal pole region of the 32-cell-stage *Xenopus laevis* embryo (Warner, Guthrie & Gilula, 1984). Controls (injection of preimmune sera, suspension buffers and an antibody to an extracellular matrix glycoprotein) had no effect on the transfer of Lucifer Yellow from cell to cell. The results of this series of dye injection experiments are summarized in Table 1 and an example of the dye transfer

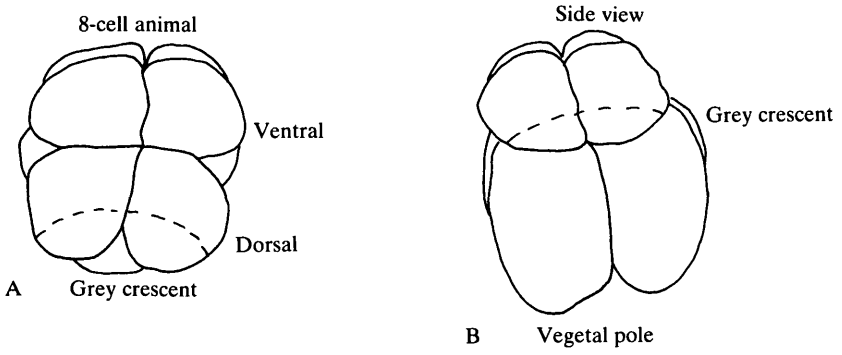

Fig. 1. Diagrams to show the location of cells at the 8-cell stage used to test developmental consequences of injection of gap junctions antibodies. (A) top view of an 8-cell-stage *Xenopus laevis* embryo. Antibody was injected into either the right-hand dorsal blastomere or the right-hand ventral blastomere. (B) 8-cell-stage embryo viewed from the grey crescent. Antibody was injected into both vegetal pole cells on the grey crescent side.

results is shown in Figure 2. In homogenates of fertilized eggs and 8-cell-stage *Xenopus laevis* embryos the antibody recognizes proteins of the same size as those found in rat liver. It may seem surprising that gap junction proteins should be found in the fertilized egg. This is probably related to the fact that early *Xenopus laevis*

Table 1. *The incidence of transfer of Lucifer Yellow at the 32-cell stage in* Xenopus laevis

Reagent	Transfer %, (n)	No Transfer % (n)
Control	69 (11)	31 (5)
Buffer 1	63 (10)	37 (6)
Buffer 2	79 (11)	21 (3)
Pre-immune A	86 (12)	14 (2)
Pre-immune B	75 (12)	25 (4)
Gap junction A	26 (13)	74 (37)*
Gap junction B	18 (9)	82 (41)*
Extacellular matrix anti-body	66 (40)	34 (21)

n = no. of injections

* incidence of transfer significantly different from controls ($P < 0{\cdot}001$, Chi-squared test).

Control values taken from Guthrie (1984).

Buffer 1 = Borate NaCl, pH 7·4.

Buffer 2 = Tris, KCl, pH 7·4.

Transfer assessed immediately after injection of Lucifer Yellow and excludes transfer to sister cell only because of persisting cytoplasmic bridges (see Guthrie, 1984).

No Transfer includes transfer to sister cell only.

The extracellular matrix antibody was raised against, and specifically interacted, with an extracellular matrix glycoprotein secreted and synthesized by rat liver and *Xenopus laevis* embryos. There was no cross-reactivity between this antibody and any of the species recognized by both gap junction antibodies.

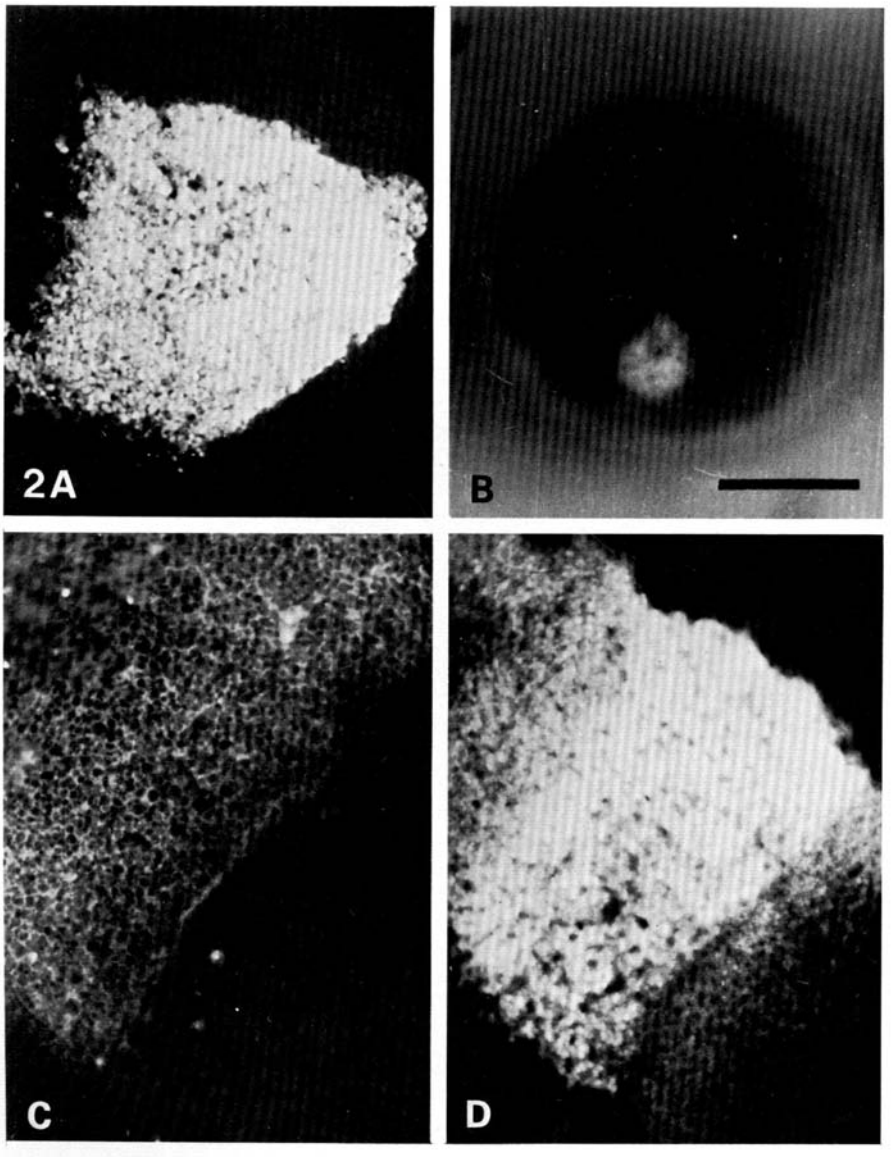

Fig. 2. Photographs of a 32-cell embryo (B) injected with gap junction antibody at the 8-cell stage and subsequently tested for the ability to transfer Lucifer Yellow out of one of the antibody containing progeny. Note dye restricted to injected cell. Scale bar = 500 μm. (A) Frozen section taken through an embryo injected with gap junction antibody at the 8-cell stage and injected with Lucifer at the 32-cell stage. Note clear restriction of Lucifer to the injected cell. Scale bar (reproduced in B) = 150 μm. (C) Frozen section through embryo injected with gap junction antibody at the 8-cell stage and subsequently (32-cell stage) stained with goat anti-rabbit FITC to reveal distribution of antibody. Note antibody spread evenly throughout cell and clear restriction at the edge of the antibody-containing region. Scale bar = 100 μm. (D) Frozen section through 32-cell-stage embryo injected with pre-immune serum at the 8-cell stage. Note extensive dye transfer to both lateral neighbours. Scale = 250 μm.

embryos carry out little *de novo* protein synthesis until they reach the 'mid blastula transition' (Newport & Kirschner, 1983). Sufficient protein must, therefore, be laid down in the oocyte to provide a store to carry the embryo through to the midblastula stage. This large, and somewhat variable, store of gap junction protein almost certainly accounts for the small number of occasions when dye transfer remained despite injection of the antibody. Some variability in the amount of antibody injected (approx. 10 nl of 1·5 mg protein ml^{-1}) undoubtedly also contributes.

MORPHOLOGICAL EFFECTS OF INJECTED ANTIBODY

In order to allow the developmental consequences of this block to communication to be assessed the antibody is injected into the dorsal, animal pole blastomere at the 8-cell stage of development (see Fig. 1A), which is destined to give rise to head ectoderm and mesoderm on the right-hand side of the tadpole (Figs 3, 4 and 5). The antibody has no effect on cleavage, and antibody-containing cells which are communication incompetent retain good resting potentials, suggesting that the presence of the gap junction antibody within the cell does not have major, deleterious metabolic effects. Electrophysiological measurements of cell-to-cell communication at intervals after injection of the antibody show that the block to communication can be maintained through at least eight rounds of cell division, which corresponds to the beginning of gastrulation (Warner, Guthrie & Gilula, unpublished). Careful observation of the embryos revealed no signs of failure of cleavage, cytolysis or death up the stage 36 (two days after injection), when the animals were scored for developmental defects. Lucifer Yellow injected at the 32-cell stage to test for communication block was still clearly visible at the time of assessment, confirming that cell death is not a major complicating factor.

Animals injected with the gap junction antibody showed a high proportion of defects in the regions derived from the antibody injected cell. In the most severely affected embryos the right eye could be completely missing and the brain was then severely retarded on the right-hand side. Mesodermal defects were reflected in the absence of anterior myotomes on the right-hand side. In less-severely affected embryos the right eye was smaller than the left and displaced from its normal position along the anteroposterior axis. The notochord was then shifted from its normal, central position towards the uninjected side of the embryo. Embryos injected with all the control reagents generated abnormalities at about the same rate as uninjected controls, with asymmetry being comparatively rare.

These defects fall into the class of patterning abnormalities where either no structure is produced, or it appears in the wrong position. The results show that interfering with direct cell-to-cell communication through gap junctions has profound consequences for development. It should therefore be possible to ascertain both when and where gap junctional communication is essential by injecting the antibody at known times and into identified cells and observing developmental consequences.

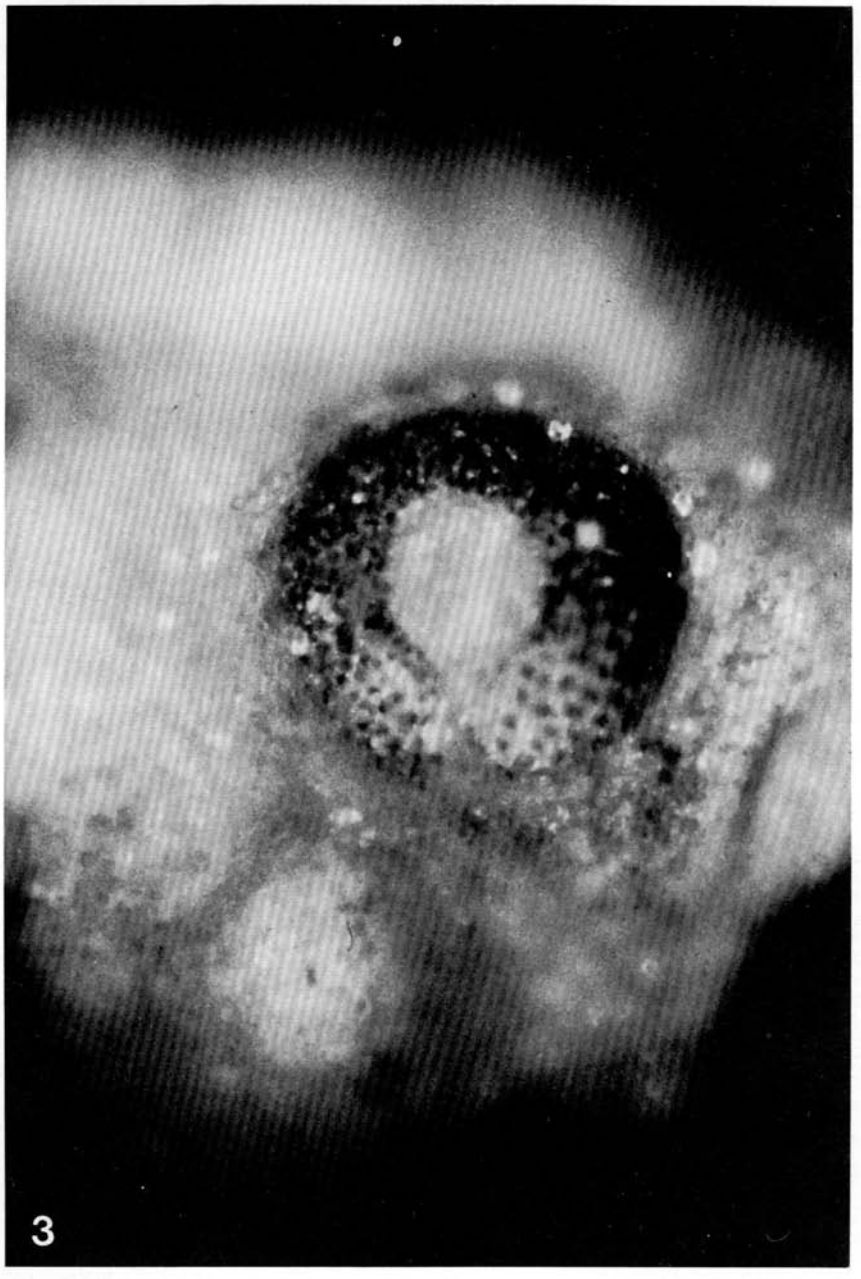

Fig. 3. Photograph of living tadpole at Nieuwkoop and Faber stage 36 taken under fluorescent optics. This tadpole was injected with Texas Red at the 8-cell stage. In this image the head structures on the right-hand side of the tadpole have been photographed. Note extensive distribution of fluorescent cells within the eyes, retina and ectoderm. Overall brightness caused by extensive labelling in the brain.

We have recently made a start on this analysis (Warner, Guthrie & Gilula, unpublished). If the antibody is injected into a ventral animal pole blastomere at the 8-cell stage (see Fig. 1A), destined to give rise to most of the ectoderm, part of

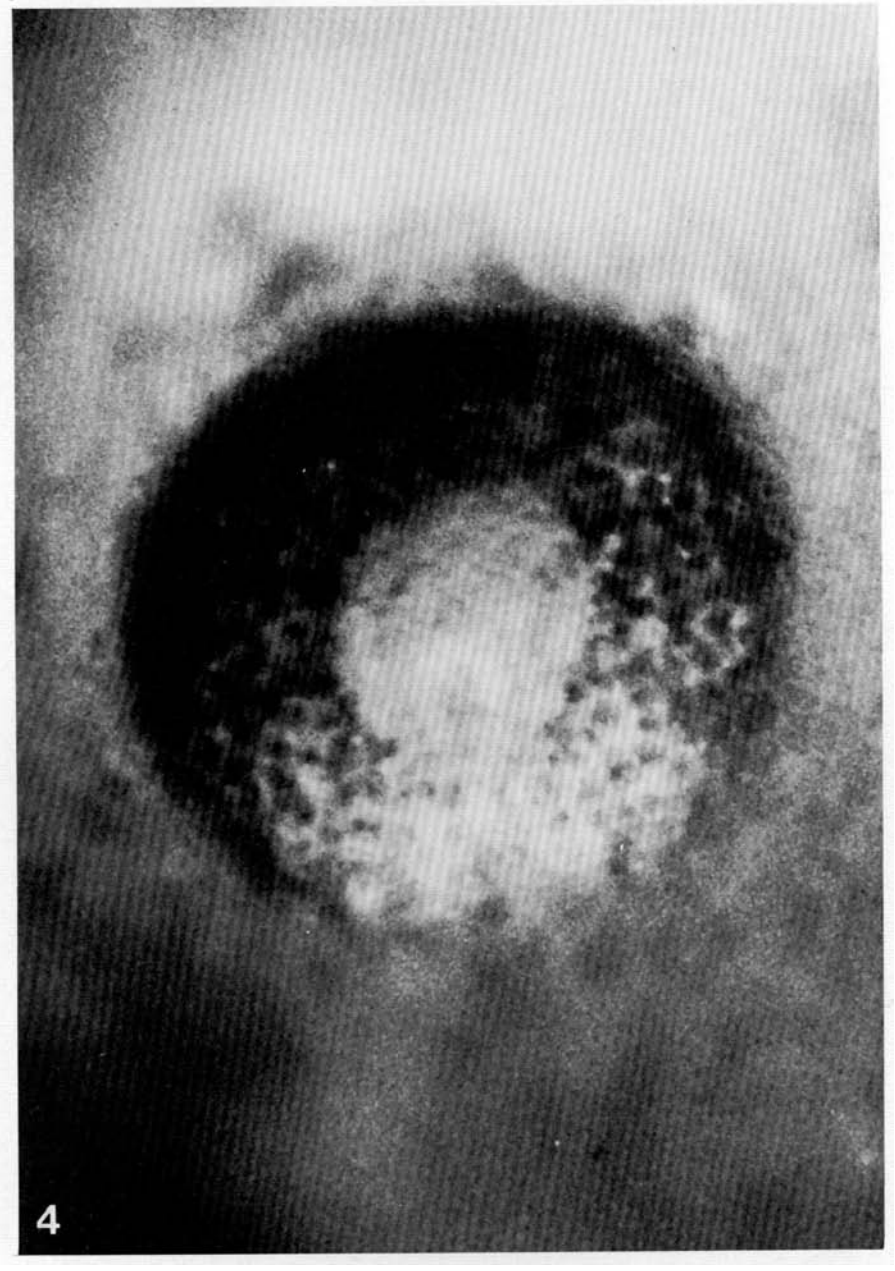

Fig. 4. Same tadpole as in Fig. 3 photographed from the left-hand side to show a clone of labelled cells in the ventral retina, illustrating the mixing across the mid-line that occurs during development of the nervous system.

Fig. 5. Same tadpole as in Figs 3 and 4. Medial clone of myotomal cells arising from the right-hand dorsal blastomere. Note coherence of mesodermal clone.

the lens and otocyst on the right-hand side and the medioventral portion of the right-hand tail myotomes (Warner, unpublished) then there is little obvious developmental effect. This is perhaps not surprising since induction of both otocyst and lens

and the formation of the tail myotomes occur after the end of the neurula stage, by which time antibody injected at the 8-cell stage is likely to have been diluted out by *de novo* synthesis of gap junction protein. It will probably be necessary to interfere with gap junction protein synthesis to affect these events. However such experiments form a useful control for the consequences of antibody injection into the dorsal animal blastomere, which has profound developmental consequences.

MESODERM INDUCTION

Recently considerable interest has focussed on the earliest developmental induction in the early amphibian embryo: induction of the mesoderm by cells lying near the vegetal pole. Nieuwkoop (1977) originally proposed that the mesoderm arises as the result of an inductive signal from the vegetal pole. This conclusion has been strengthened by experiments by Gurdon *et al.* (this volume) demonstrating that recombinants of vegetal pole and animal pole cells induce activation of the actin gene in the animal portion of the recombinant. Correlated experiments have been carried out by Gimlich & Gerhart (1984) who took advantage of the axis deficiencies generated by u.v. irradiation of the fertilized egg. Embryos from irradiated batches of eggs develop a variety of axis deficiencies ranging from mild defects such as microcephaly to severe defects where no mesoderm, and therefore no primary axis, is formed at all. These embryos contain endoderm cells surrounded by ectoderm alone. Gimlich & Gerhart showed that u.v.-irradiated embryos could be rescued from axis deficiency by the transplantation of dorsal blastomeres from the vegetal pole at the 64-cell stage. The factor responsible for inducing the formation of the mesoderm sometimes lay in the vegetal most blastomeres and sometimes in those of the next tier of cells up (see Gimlich, this volume). Gimlich & Gerhart presumed that the induction of the mesoderm involved some cellular interaction. We have recently (Warner, Guthrie & Gilula, unpublished) examined whether direct cell-to-cell communication via gap junctions might be involved by injecting the gap junction antibody into both dorsal, vegetal pole cells at the 8-cell stage (Fig 1B). We chose to inject at this stage in order to be certain that the antibody was present in all cells likely to contain the inducing factor. Control injections were made of pre-immune serum. The antibody-injected embryos developed with a range of axis defects, as after u.v. irradiation. The complete range of axis defects from microcephaly through absence of all head structures to complete axis deficiency was seen. Some of the embryos injected with pre-immune serum also developed axis deficiencies, almost certainly because of damage at the base of the vegetal pole, causing deletion of those cells containing the mesoderm-inducing material. Out of 43 embryos injected with gap junction antibody 77 % had partial or complete axis deficiencies, compared to 30 % ($n = 23$) of controls injected with pre-immune serum. These results are highly significantly different ($P < 0{\cdot}001$, Chi-squared test) and suggest rather strongly that the induction of mesoderm by dorsal vegetal pole cells takes place through gap junctions.

In summary, the advent of antibodies to gap junction protein, which completely block transfer of materials through gap junctions, is now making it possible to dissect out the contribution of direct cell-to-cell communication to the various cellular interactions which take place during early embryonic development. By injecting such antibodies at different times and into identified cells it should be possible to monitor precisely when and where gap junctions play an essential part in the poorly understood processes of spatial and temporal differentiation. The antibodies should also make it possible to determine which part of the molecules comprising the gap junction are involved in the functional control of gap junction permeability.

I am indebted to my colleagues Sarah Guthrie and N. B. Gilula for allowing me to quote the results of unpublished collaborative experiments. I am grateful to the Medical Research Council and the Wellcome Trust for support.

REFERENCES

ARMSTRONG, D., TURIN, L. & WARNER, A. E. (1983). Muscle activity and the loss of electrical coupling between striated muscle cells in *Xenopus laevis* embryos. *J. Neurosci.* **3**, 1414–1421.

BLACKSHAW, S. E. & WARNER, A. E. (1976). Low resistance junctions between mesoderm cells during formation of trunk muscles. *J. Physiol.* **255**, 209–230.

BLENNERHASSETT, M. & CAVENEY, S. (1984). Separation of developmental compartments by a cell type with reduced junctional permeability. *Nature* **309**, 361–364.

DIXON, J. S. & CRONLEY-DILLON, J. R. (1972). The fine structure of the developing retina in *Xenopus laevis*. *J. Embryol. exp. Morph.*, **28**, 659–666.

DIXON, J. S. & CRONLEY-DILLON, J. R. (1974) Intercellular gap junctions in pigment epithelium during retinal specification in *Xenopus laevis*. *Nature* **251**, 505.

GIMLICH, R. L. & GERHART, J. C. (1984). Early cellular interactions promote embryonic axis formation in *Xenopus laevis*. *Devl Biol.* **104**, 117–130.

GUTHRIE, S. G. (1984). Patterns of junctional communication in the early amphibian embryo. *Nature* **311**, 149–151.

ITO, S. & LOEWENSTEIN, W. R. (1966). Ionic communication between early embryonic cells. *Devl Biol.* **19**, 228–243.

JACOBSON, C-O. (1964). Motor nuclei, cranial nerve roots and fibre pattern in the medulla oblongata after reversal experiments on the neural plate of axolotl larvae. *Zool. Biddrag. Uppsala.* **36**, 73–160.

KEETER, J., PAPPAS, G. & MODEL, P. (1975). Inter- and intra-myotomal gap junctions in the Axolotl embryo. *Devl Biol.* **45**, 21–34.

LAWRENCE, P. A. (1973). A clonal analysis of segment development in *Oncopeltus*. *J. Embryol. exp. Morph.* **30**, 681–699.

LOCKE, M. (1959). The cuticular pattern in an insect *Rhodnius prolixus*. *J. exp. Biol.* **36**, 459–477.

NEWPORT, J. &KIRSCHNER, M. (1982). A major developmental transition in early *Xenopus* embryos: 1. Characterization and timing of cellular changes at the mid-blastula stage. *Cell* **30**, 675–686.

NIEUWKOOP, P. D. (1977). Origin and establishment of embryonic polar axes in amphibian development. *Curr. Top. devl Biol.*, **11**, 115–132.

PALMER, J. F. & SLACK, C. (1970). Some bioelectric parameters of embryos of *Xenopus laevis*. *J. Embryol. exp. Morph.* **24**, 535–553.

POTTER, D. D., FURSHPAN, E. J. & LENNOX, E. S. (1966). Connections between cells of the developing squid embryo as revealed by electrophysiological methods. *Proc. natn. Acad. Sci., U.S.A.*, **55**, 328–333.

SHERIDAN, J. D. (1968). Electrophysiological evidence for low resistance electrical connections between cells of the chick embryo. *J. Cell Biol.* **37**, 650–659.

SIMPSON, I., ROSE, B. & LOEWENSTEIN, W. R. (1977). Size limit of molecules permeating the junctional membrane channels. *Science* **195**, 294–396.

WARNER, A. E. (1973). The electrical properties of the ectoderm during induction and early development of the nervous system. *J. Physiol.*, **235**, 267–286.

WARNER, A. E. (1979). Consequences of cell to cell signals in the early embryo. In *The Role of Intercellular Signals: Navigation, Encounter, Outcome.* (ed. J. G. Nicholls). pp. 179–200. Berlin: Verlag Chemie.

WARNER, A. E. (1981). The early development of the nervous system. In *Development in the Nervous System. Brit. Soc. devl. Biol.* Symposium **5**, (ed. D. Garrod & J. Feldman). pp. 109–127. Cambridge: University Press.

WARNER, A. E. & LAWRENCE, P. A. (1973). Electrical coupling across developmental boundaries in insect epidermis. *Nature* **245**, 47–49.

WARNER, A. E. & LAWRENCE, P. A. (1982). Permeability of gap junctions at the segmental border in insect epidermis. *Cell* **28**, 243–252.

WARNER, A. E., GUTHRIE, S. C. & GILULA, N. B. (1984). Antibodies to gap junctional protein selectively disrupt junctional communication in the early amphibian embryo. *Nature* **311**, 127–131.

DISCUSSION

Speaker: Anne Warner (UCL)

Question from Elizabeth Jones (Warwick):
I wonder if you have used as a control an antibody against a cytoplasmic antigen, because the control antibody you are using is against an extracellular matrix component, and this will obviously not come into contact with its antigen. As a follow-up to that, have you tried using the antigen/antibody complex injected into blastomeres as a control?

Answer:
We have not used an antigen/antibody complex – that's a control we would like to do. The problem with using an antibody against a cytoplasmic component is that of finding one which does not itself have consequences for development. Most of the antibodies that have been accessible to us have been against cytoskeletal components and we have been very reluctant to use those because cytoskeletal elements may themselves have important functions during development. There is now a monoclonal antibody against the gap junction protein which apparently is not functionally effective and we are hoping to get this to use as a control.

Question from J. Pitts (Glasgow):
For this approach it is essential to have an inhibitor which is specific. Now, the purified fraction which you have reacts with the 54K protein and the 27K protein. There are two possible explanations for this, either these proteins are the same, as you suggest, or else they are different and the antiserum is not specific. Do you have good independent evidence, such as peptide maps, that these two proteins are really the same?

Answer:
We have not done any peptide mapping as yet. We are fairly convinced that they are related proteins, for the reason that the proportions of 54K and 27K obtained in the preparations can be shifted by varying the degree of proteolysis. Also antibody removed from the 54K fraction and applied to the 27K protein still binds. Both these results are unpublished data from C. R. Green and N. B. Gilula. That is clearly not an unequivocal answer and I suspect that this is not going to be an easy thing to resolve because one always has the problem, when using a polyclonal antiserum, that there may be minor contaminants which are actually doing the job that you think your major protein is doing. I think that without a functionally effective monoclonal antibody, that question is never going to be resolved properly.

Pitts:
You talk a lot about the clone of communication-incompetent cells, suggesting that you know how long the inhibition of the junctions lasts. Do you have evidence that these cells do not die after two or three divisions, and have you done Texas Red injections along with the antibody to show where these cells finish up?

Answer:
We can be pretty confident that they don't die within two or three rounds of cell division because if one watches the embryos, one can see them go through cleavage divisions throughout development. As a control, it is possible to inject Lucifer Yellow on its own, and then produce damage by over-irradiating with u.v. light. When one does that, the cell starts to slow its cleavage rate, and eventually cytolyses and is extruded from the embryo. We never see this effect with the antibody-injected embryos. I should also have mentioned that the Lucifer Yellow that we use to test for transfer is still present in the embryo three days later. So, I don't think that embryo cell death is a problem although it is something that we have thought about a lot.

I think the length of time that electrical communication remains blocked does vary from embryo to embryo, depending on how much antibody has been injected and how much gap junction protein is present as a store. However we know that the block can be effective for *at least* eight rounds of cell division, which takes the embryo up to the early gastrula stage. Clearly it is going to be important to map the length of time that the antibody remains effective in individual embryos. Unfortunately, the only unequivocal way to do that is with electrical communication and this requires that you break the seal into the blastocoel, so it is not really possible to follow the same embryo as it grows up.

Pitts:
You have not put Texas Red into the injected cells?

Answer:
No, that is an experiment we are going to do. At the moment, I am doing some control injections in order to build up a normal lineage pattern and shall then do the lineage along with the antibody, although, as I say, the Lucifer Yellow which is injected into cells which contain the antibody is still there three days later.

Question from C. Wylie (St Georges):
With regard to specificity of the antibody action it seems to me that one thing you could do is to inject an antibody against various cytoplasmic constituents and then at least show whether or not they block the transfer of ions. You may well be right that if you inject anti-actin you will not do development any good, but at least you could show that the blockage of coupling is specific.

Answer:
Obviously you are right. For the moment, we have stuck with the pre-immune sera and the anti-extracellular matrix antibody which is not the ideal control. I think one has to do a whole series of further controls to resolve that question unequivocally.

Question from E. deRobertis (Basel):
I wonder if you would care to comment about the use of anti-sense oligonucleotides?

Answer:
We have been doing some experiments using anti-sense oligonucleotides prepared on the basis of partial sequences of the gap junction protein. I have not included those experiments because they are still at a very early stage and we have not yet got the sense oligonucleotides to use as a control. Also, we are not yet at all clear about the fate of the oligonucleotide that we inject. With these caveats I should say that the anti-sense oligonucleotides do have effects which are rather similar to those of the anti-gap junction antibody. They do not, however, block transfer at the 32-cell stage, and this is presumably because they only inhibit *de novo* synthesis of the gap junction protein, rather than the activity of the maternal store.

Question from H. Woodland (Warwick):
I would like to ask about the unilateral nature of the response. In the dorsal animal injections at the 8-cell stage, was the defective nervous system formed from the cells on the side that was injected?

Answer:
Yes, we think so. Some of those cells must come from the antibody-injected region because one can still see the Lucifer Yellow in there, but that is not an unequivocal answer because it may well be that a larger proportion comes from the other side than it would normally. I think we really have to try and sort out the whole question of what a clone of communication-incompetent cells does, not only to the cells which are incompetent, but also to the cells that are still able to communicate and whether the whole system shifts as a result.

Question from Mae Wan Ho (Open University):
You mentioned that the gap junctions allow both small molecules and ions to pass through. Which do you think is the primary factor disturbing pattern formation, interruption of electrical coupling or of small molecule transport?

Answer:
We don't know. The problem is that we have not yet been able to test whether the dye transfer block occurs at a lower level of antibody than the electrical communication block. All I can tell you is that embryos which have been scored for failure to transfer dye also fail to transfer small ions.