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# Expression of a dominant negative inhibitor of intercellular communication in the early *Xenopus* embryo causes delamination and extrusion of cells

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## SUMMARY

**A chimeric construct, termed 3243H7, composed of fused portions of the rat gap junction proteins connexin32 (Cx32) and connexin43 (Cx43) has been shown to have selective dominant inhibitory activity when tested in the *Xenopus* oocyte pair system. Co-injection of mRNA coding for 3243H7 together with mRNAs coding for Cx32 or Cx43 completely blocked the development of channel conductances, while the construct was ineffective at blocking intercellular channel assembly when coinjected with rat connexin37 (Cx37). Injection of 3243H7 into the right anterodorsal blastomere of 8-cell-stage *Xenopus* embryos resulted in disadhesion and delamination of the resultant clone of cells evident by embryonic stage 8; a substantial number, although not all, of the progeny of the injected cell were eliminated from the embryo by stage 12. A second construct, 3243H8, differing from 3243H7 in the relative position of the middle splice, had no dominant negative activity in the oocyte pair assay, nor any detectable effects on *Xenopus* development, even when injected at four-fold higher concentrations. The 3243H7-induced embryonic defects could be rescued by coinjection of Cx37 with 3243H7. A blastomere reaggregation assay was used to demonstrate that a depression of dye-transfer could be detected in 3243H7-injected cells as early as stage 7; Lucifer yellow injections into single cells also demonstrated that injection of 3243H7 resulted in a block of intercellular communication. These experiments indicate that maintenance of embryonic cell adhesion with concomitant positional information requires gap junction-mediated intercellular communication.**

Key words: gap junction, intercellular communication, dominant-negative mutation, connexin

## INTRODUCTION

In most animal tissues, intercellular channels present in gap junctions allow the passive diffusion of ions and small water soluble molecules from cell to cell (Loewenstein, 1981). These channels are unusual in their relative nonselectivity and large diameter (Simpson et al., 1977; Schwartzman et al., 1981). They are composed of members of the connexin family of proteins (Paul, 1986; Kumar and Gilula, 1986; Beyer et al., 1987; Zhang and Nicholson, 1989; Hoh et al., 1991; Hennemann et al., 1992a,b; Paul et al., 1991; Haefliger et al., 1992; White et al., 1992). Connexins oligomerize in the *trans*-Golgi to form half-channels (Musil and Goodenough, 1993), termed connexons, which span a single lipid bilayer. To form a complete intercellular channel spanning two membranes, connexons in adjacent cells associate to define an axial, hydrated pore (Caspar et al., 1988) allowing diffusional movement of molecules up to about $1\times10^3$ $M_r$, depending on the type of connexin present (Verselis and Brink, 1986; Steinberg et al., 1994).

In excitable tissues, the significance of gap junctional communication is clear. At electrical synapses in the nervous system, gap junctional intercellular communication allows rapid propagation of ionic current, both determining the dynamic properties of certain neuronal assemblies and regulating the ionic environment in which neuronal activities occur (Jaslove and Brink, 1986; Tessier-Lavigne and Attwell, 1988; DeVries and Schwartz, 1989). Gap junctional communication may also be an initial step in the establishment of other types of synapses, such as the neuromuscular junction (Allen and Warner, 1991). In non-excitable cells, the functional significance of communication is not clear, although gap junctions are likely to play diverse roles in cell growth control (Loewenstein and Rose, 1992) and the propagation of inductive or positional signals during development (Guthrie and Gilula, 1989). Gap junctions may promote transduction of signals within tissues since they are permeable to second messengers such as cAMP, inositol phosphates and $Ca^{2+}$ ions (Lawrence et al., 1978; Boitano et al., 1992; Murray and Fletcher, 1984; Sáez et al., 1989).

Substantial correlative evidence suggests a role for junctional communication in embryonic development of the frog *Xenopus laevis*. Cells of the early *Xenopus* embryo are joined by gap junctions (Palmer and Slack, 1970) and changes in the pattern of junctional communication occur during episodes of embryonic induction and tissue formation (Blackshaw and

Warner, 1976; Warner, 1973). The pattern of dye transfer between embryonic blastomeres is not uniform even during early cleavage stages. At the 32-cell stage in *Xenopus*, dye transfer activity is related to the plane of bilateral symmetry, and is stronger and more frequently detected among animal hemisphere blastomeres near the future dorsal midline than among ventral cells (Guthrie, 1984). The pattern of communication is altered by treatments that cause reduction or duplication of the notochord and other dorsal axial structures. For example, lithium chloride treatment of the early embryo causes improper commitment of ventral equatorial cells to dorsal mesodermal fates, leading to twinning or more severe abnormalities (Nagajski et al., 1989; Kao and Elinson, 1989; Busa and Gimlich, 1989). This is accompanied by an increased frequency of Lucifer yellow transfer in the ventral half of the embryo. Conversely, irradiating the vegetal surface of the uncleaved zygote with ultraviolet light reduces the completeness of the mesodermal structures that develop from the dorsal half of the embryo (Scharf and Gerhart, 1983). Irradiated embryos show decreased dye transfer activity in the dorsal quadrant at the 32-cell stage (Nagajski et al., 1989). Thus, ventralization correlates with reduced dye transfer, while increased dye transfer follows dorsalization. In the one exception to this, Guthrie et al. (1988) treated embryos with the weak base methylamine, which increased cytoplasmic pH and led to increased ventral dye transfer after a short treatment. Embryos cultured for longer periods in methylamine developed normally; the duration of the pH rise and effect on gap junctions were not determined.

Communication and patterning were also linked in a study by Olson et al. (1991), who found that axial duplication in *Xenopus* caused by misexpression of the gene *Xwnt8* was accompanied by an increase in ventral cell communication. *Xwnt8* mRNA normally begins to accumulate in prospective ventroposterior mesoderm during gastrulation (Christian et al., 1991). In embryos inappropriately expressing *Xwnt8* at the blastula stage, the frequency of detectable ventral dye transfer is increased and the normal dorsoventral gradient in dye coupling is eliminated. Other factors, such as activin B, that dorsalize prospective ventral tissue in the embryo when they are inappropriately expressed (Thomsen et al., 1990), also elicit increased ventral dye transfer as an early response (Olson and Moon, 1992).

Further evidence of a direct role for communication in embryogenesis and cellular differentiation comes from perturbation with anti-connexin antibodies (Warner et al., 1984; Lee et al., 1987; Fraser et al., 1987). Some antisera produced against rat Cx32 cross-react with proteins in extracts from the *Xenopus* early embryo. When microinjected into embryonic cells in the frog and the mouse, such antisera reduce ionic conductance or transfer of tracer dyes (Warner et al., 1984; Lee et al., 1987). In *Xenopus*, when the injected cells include precursors of the anterior central nervous system, abnormalities in the development of the brain, eye and other anterior structures result (Warner et al., 1984). These results indicate the involvement of junctional communication in any of a series of tissue interactions, since a block to communication is present throughout a prolonged and ill-defined period.

We have employed a dominant-negative connexin mutation specifically to inhibit communication. RNA encoding the inhibitor was injected into anterodorsal blastomeres of the 8-cell-stage embryo. Delamination of surface ectoderm was evident by stage 8 and a substantial number, although not all, of the progeny of the injected cell was eliminated from the embryo by stage 12. This behavior was distinctly different from the results of antibody blockade, although in both cases, loss of derived structures in tadpole-stage embryos was observed. Since similar types of delamination have been observed in experiments in which cadherin-based intercellular adhesion was depressed, our data suggest that gap junction-mediated communication may modulate intercellular adhesion.

## MATERIALS AND METHODS

### Construction of chimeric connexins 3243H7 and 3243H8

Two four-part chimeric connexins were produced (Fig. 1). The first, termed 3243H7, resulted from the fusion of N- and C-terminal portions of previously reported two-part chimeras (N-terminal from 3243H4; C-terminal from 3243H1) as described by Bruzzone et al. (1994). To produce precise two-part splices, Cx32 and C43 were cloned in tandem into Bluescript KS+ (Stratagene, LaJolla CA) and an oligonucleotide-directed deletion of the intervening sequences was performed according to Eghtedarzadeh and Henikoff (1986). To construct the four-part 3243H7, 3243H4 and 3243H1 were placed in tandem. The sequence of the oligonucleotide used to direct this splice was 5′-CTC ATC CAG TGG TAC CTC TAC CCG GGC TAT-3′. The amino acid sequence of 3243H7 contains residues 1-96 from Cx32, residues 98-175 from Cx43, residues 156-208 from Cx32 and residues 229-382 from Cx43. In order to create the four part chimera designated 3243H8, we used a polymerase chain reaction (PCR) based method which allows gene splicing by overlap extension, as described in detail by Bruzzone et al. (1994). The amino acid sequence of 3243H8 contains residues 1-96 from Cx32, residues 98-150 from Cx43, residues 131-208 from Cx32 and residues 229-382 from Cx43. The sequence of all chimeric proteins was verified with Sequenase® (United States Biochemical, Cleveland, OH), following protocols recommended by the manufacturer.

### *Xenopus* oocyte injections and electrophysiological measurements

The coding sequence of 3243H7 was subcloned into the *Bgl*II site of the RNA expression vector SP64T, in between the 5′ and 3′ non-coding regions of *Xenopus* β-globin (Krieg and Melton, 1984). Recombinant plasmids were linearized with either *Bam*HI or *Xba*I (New England Biolabs, Beverly, MA) gel purified and used as templates (Maniatis et al., 1982). In vitro synthesis of capped RNA was carried out with SP6 polymerase (mMessage mMachine, Ambion, Austin TX), according to the manufacturer's instructions. For 3243H8, the final PCR product contained an SP6 polymerase promoter so RNA was produced directly from the DNA fragment without subcloning or restriction digestion.

Oocytes were collected from *Xenopus laevis* females and processed for the paired *Xenopus* oocyte expression assay as described (Swenson et al., 1989). To eliminate the possible contribution of endogenous intercellular channels to the measured conductance, manually defolliculated oocytes were injected with an antisense oligonucleotide corresponding to a portion of the coding sequence of *Xenopus* connexin38 (Cx38) (Bruzzone et al., 1993; 3 ng/oocyte, 5′-CTGACTGCTCGTCTGTCCACACAG-3′). Following overnight incubation at 18°C, each antisense-treated oocyte was then injected with 40 nl of connexin RNA (10-100 pg), manually stripped of the vitelline envelope and paired for quantitation of junctional communication by double voltage clamp (Spray et al., 1981). Both cells were initially clamped at –40 mV to ensure zero transjunctional potential and therefore zero junctional current. For simple measurement of con-

ductance levels, alternating symmetrical depolarizing steps of 10-20 mV were imposed. The resulting junctional current is equal in magnitude to the current required to maintain the membrane potential of the unstepped cell. Junctional conductance is equal to the junctional current divided by the transjunctional voltage step.

### *Xenopus* embryo preparation and injections

Production of eggs, fertilization and removal of jelly coat were performed according to Newport and Kirschner (1982). Embryos were staged according to Nieuwkoop and Faber (1967). In most experiments, the right anterodorsal blastomere was injected at the 8-cell stage. Anterior cells were identified on the basis of the fixed orientation of the embryo with respect to gravity. Lack of pigmentation was used as a marker to identify the dorsal cells which is 70 to 80% accurate (Moody, 1987). Embryos without clear differences in pigmentation between anterior cells were discarded. In some experiments, all cells of 4-cell-stage embryos were injected. Injections consisted of 2-4 nl of water containing RNA at 100-200 ng/µl. Injection pipettes were pulled (Model 730, David Kopf Instruments, Tujunga CA) and broken to a tip diameter of <10 µm under a compound microscope. Pipettes were individually calibrated after loading RNA solutions by injecting into oil, measuring the diameter of the drop and adjusting the time of the injection pressure pulse to achieve the desired volume (Picospritzer II, General Valve Corp, Fairfield NJ). Embryos were placed in MB/10 (Modified Barth's solution diluted 1:10) containing 5% Ficoll for injection and maintained in this medium at 16°C until late blastula when they were returned to medium without Ficoll.

### Lineage tracing

Lineage tracing was carried out using either rhodamine-dextran according to Gimlich (1991) or colloidal gold-BSA as described by Niehrs and DeRobertis (1991). Following whole-mount silver enhancement of colloidal-gold, embryos were embedded in paraffin for sectioning.

### Dye coupling measurements

To prepare donor cells, embryos were manually stripped of vitelline envelopes and the vegetal cells were removed with an eyelash, leaving intact animal caps. Caps were incubated with 0.2 mg/ml 6-carboxyfluorescein diacetate (Sigma, St. Louis MO) in full-strength Modified Barth's (MB) for 20 minutes at room temperature then washed three times for 2 minutes by transferring the caps to successive dishes of fresh MB. Recipient cells were prepared from embryos co-injected with water or 3243H7 RNA and rhodamine-dextran (Sigma; 4 nl of 2.5 mg/ml). All four anterior blastomeres of 8-cell-stage embryos were injected. Donor and recipient caps were mixed and the cells were dissociated by gentle triturition through a fire-polished Pasteur pipette in MB without $Ca^{2+}$ according to Gurdon (1988). The dissociated cells were transferred with a minimal amount of MB to agarose-coated Eppendorf tubes containing 1 ml of Liebovitz medium, 10% calf serum, 30% water, centrifuged for 6 minutes at 100 ***g*** and incubated at room temperature for 2.5 hours. The developmental stage of undissected sibling embryos at the beginning of the incubation was scored as the relevant developmental stage. Following the incubation, the pellet was resuspended in MB without $Ca^{2+}$ to facilitate complete disaggregation and prevent reformation of gap junctions. The cell suspension was analyzed for dye transfer by fluorescence microscopy (Zeiss Axioskop, Thornwood NJ). Cells labeled with both rhodamine and fluorescein were scored as having established junctional communication.

### Dye injections in embryos

8-cell embryos were injected in the RAND cells with either 3243H7 or 3243H8 together with the rhodamine-dextran lineage tracer as before, cultured at 18°C until stage 7, devitellinized with #5 Dumont forceps and transferred to 35 mm Petri dishes with a 1% agarose bottom layer to keep the embryos from sticking. The embryos were then transferred to the stage of a Zeiss microscope and the rhodamine-labeled clone visualized with fluorescence optics and a 10× objective. Switching to the fluorescein filter set, the tip of a microelectrode filled with 1 µl 4% Lucifer yellow in 0.1% LiCl, then back-filled with 3 M LiCl was visualized, then placed near the boundary of the rhodaminated clone. The electrode was inserted intracellularly and only cells with stable resting potentials >20 mV were iontophoretically injected with LY with 100 ms, 10 nA pulses at 1/sec. Injections were continued as long as stable resting potentials were held (1-10 minutes), then the embryos were fixed in 1% formaldehyde made fresh from paraformaldehyde in 1% PBS for 1 hour. Embryos were then frozen in Freon slush and serially frozen sectioned. Frozen sections were examined on a Zeiss Axioskop and fluorescent images recorded on Kodak Tmax film.

### Western blotting

Embryos were suspended in lysis buffer consisting of 5 mM Tris pH 8.0, 5 mM EDTA, 5 mM EGTA with 1/1000 volume of Trasylol (FBA Pharmaceuticals, West Haven CT) and 1/1000 volume of a mixture of protease inhibitors (chymostatin, leupeptin and pepstatin at 10 mg/ml). Embryos were homogenized by passing through a 1.5 inch, 22-gauge needle 15 times. 10-20 embryos were processed in 750 µl of lysis buffer. Lysates were spun at 3000 ***g*** at 4°C for 5 minutes and the pellet containing yolk platelets was discarded. The supernatant was spun at 100,000 ***g*** for 30 minutes to collect membranes and the pellet was resuspended in lysis buffer at 3 embryos/10 µl. SDS sample buffer was added and 0.5-1 embryo equivalents were loaded per lane of a 11% polyacrylamide mini-gel (Bio-Rad, Richmond CA). After separation, proteins were transferred to the hydrophobic side of Immobilon-P membranes (Millipore, Medford MA) using a tank blotter with plate electrodes (Bio-Rad) at 65 volts for 30 minutes with precooled transfer buffer (4°C) with 10% methanol. No additional cooling was utilized. Transferred proteins were visualized directly on the hydrophobic surface and the position of molecular weight standards marked with a needle. Membranes were blocked with 0.2% defatted casein (Tropix, Bedford MA) in PBS containing 0.5% Tween 20 (blocking buffer) for 1 hour at room temperature. Primary antibodies were diluted in blocking buffer and incubated with the membranes for 1-2 hours at room temperature. For 3243H7 and 3243H8, a 1/10,000 dilution of an anti-Cx43 affinity-purified antibody was used (Beyer et al., 1989); for Cx37, an anti-Cx37 affinity-purified antibody was diluted 1/5000 (Goliger and Paul, 1994). Membranes were washed 3 times for 10 minutes with blocking buffer on a shaking table and incubated with a 1/10,000 dilution of alkaline-phosphatase-conjugated goat anti-rabbit IgG (Promega, Madison WI) for 1 hour at room temperature. Membranes were washed as described above and color developed as specified by the manufacturer of the secondary antibody.

## RESULTS

### 3243H7 but not 3243H8 is a dominant-negative inhibitor of communication in oocyte pairs

A series of chimeric connexins composed of rat Cx32 and Cx43 had previously been constructed (Bruzzone et al., 1994). Chimeras that did not result in detectable intercellular communication were screened for potential dominant-negative activity. A diagram of the composition and topological orientation of two chimeras, relative to their parent connexins, is presented in Fig. 1. The limits of the various transmembrane, extracellular and cytoplasmic regions were attributed following Kyte-Doolittle hydropathicity plots, as described in Paul (1986) and Beyer et al. (1987). It should be emphasized

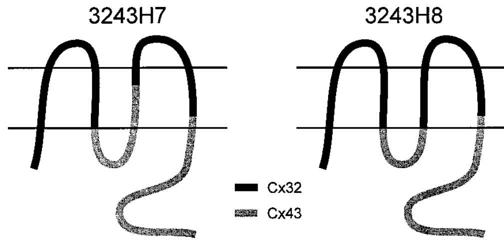


**Fig. 1.** Topological models of chimeric connexins 3243H7 and 32437H8. Both are four-part chimeras consisting of domains from rodent Cx32 and Cx43. They are identical except that the position of the middle splice was moved by 25 amino acids.

**Table 1. Conductances measured between oocyte pairs injected with connexin mRNAs**

| mRNAs injected into oocytes (cell 1/cell 2) | $G_j$ [µs] | Number |
|---|---|---|
| $[Cx32]^2$ | 20.2 | 10 |
| $[Cx37]^2$ | 5.5 | 7 |
| $[Cx43]^2$ | 16.5 | 3 |
| XCx38 / Cx43 | 8.4 | 19 |
| [3243**H7**]$^2$ | 0.0 | 6 |
| [3243**H8**]$^2$ | 0.0 | 6 |
| [3243**H7**+Cx32]$^2$ | 0.1 | 12 |
| [3243**H7**+Cx37]$^2$ | 6.4 | 7 |
| [3243**H7**+Cx43]$^2$ | 0.0 | 7 |
| 3243**H7**+XCx38 / Cx43 | 0.0 | 13 |
| [3243**H8**+Cx32]$^2$ | 18.4 | 6 |
| [3243**H8**+Cx43]$^2$ | 19.0 | 6 |

Individual oocytes were injected with the listed mRNAs then paired. If both oocytes received the same connexin, e.g. Cx32, this is shown as $[Cx32]^2$. If the oocytes received different connexins, e.g. XCx38 and Cx43, this is shown as XCx38 / Cx43.

that these are approximate borders, since this analysis leaves some ambiguity as to the precise location where the polypeptide chain leaves or enters the plasma membrane.

We tested the ability of the chimeras to interfere with junctional communication by co-expressing them with normal connexins in a paired *Xenopus* oocyte expression system (Dahl et al., 1987; Swenson et al., 1989). Oocyte pairs expressing rat Cx32, Cx37 or Cx43 developed high levels of conductance (Table 1). In contrast, pairs expressing either 3243H7 or 3243H8 did not exhibit conductances above a background level even though the oocytes produced levels of protein equal or greater in magnitude to other connexins (data not shown). When 3243H7 mRNA was co-injected with mRNA for Cx32 or Cx43, no conductance above background was observed, indicating dominant-negative activity. However, 3243H7 was not able to inhibit communication produced by Cx37. Thus, the ability of 3243H7 to interfere with the development of communication appeared to be connexin-specific. 3243H8 did not inhibit communication when co-expressed with either Cx32 or Cx43. Since 3243H8 differs from 3243H7 only in the relative position of the middle splice (Bruzzone et al., 1994), 3243H8 can serve as an excellent negative control for in vivo studies.

## Expression of 3243H7 but not 3243H8 in the *Xenopus* embryo results in a developmental defect

The right anterodorsal blastomere (RAND cell) of 8-cell-stage *Xenopus* embryos was injected with RNAs encoding chimeric connexins (Fig. 2A). In embryos injected with 3243H7 RNA, no changes in the pattern of cleavage were observed during early stages (Fig. 2B). However, regionally localized lesions appeared in the ectoderm during gastrulation (Fig. 2C, arrows). In a typical experiment, these lesions appeared in 70-80% of the injected embryos. We assume that these percentages reflect errors incurred by assigning dorsoventral polarity on the basis of asymmetric pigment distribution at the 8-cell stage (Moody, 1987). Intentional injection of 3243H7 into ventral cells results in a normal phenotype (data not shown) which is consistent with reduced levels of intercellular communication between ventral cells during early *Xenopus* development (Guthrie, 1984). These lesions were never seen in similar stage embryos injected with 3243H8 (Fig. 2D). The lesions seen in Fig. 2C were similar in appearance to defects produced by ectopic expression of high levels of N-cadherin or dominant-negative inhibitors of cadherin activity (Detrick et al., 1990; Kintner, 1992; Levine et al., 1994). The appearance of the blastopore and ventral cells were normal. Just before hatching, the mass of extruded material remained evident and some anterodorsal features appeared to be attenuated on the side of the injection (Fig. 2E). At tadpole stages, the loss of anterodorsal structures was more evident, e.g. right but not left eye, (Fig. 2F, tadpoles indicated by circled arrow). RNA injections did not interfere with or alter the timing of cell divisions, at least during early stages where such changes are readily apparent (data not shown). The specificity of the dominant-negative effect was demonstrated by injecting embryos with 3243H8, which does not affect intercellular communication as shown above. Embryos injected with 3243H8 did not develop lesions (Fig. 2D) or loss of anterodorsal structures (Fig. 2F, tadpoles not indicated by arrow).

Embryos injected with 3243H7 and 3243H8 RNAs were subjected to western blot analysis demonstrating that each was efficiently expressed (Fig. 3). 24 embryos were injected with each chimeric RNA and 8 of each type were harvested for blotting at stage 8, before gross defects could be observed. The remaining embryos were allowed to develop to score the phenotype. 80% of the remaining embryos expressing 3243H7 developed a defect; embryos expressing 3243H8 were normal; however, a small number (typically 1-3%, see Table 2) showed defects qualitatively dissimilar to those produced by 3243H7. In this experiment, embryos expressed an epitope-tagged version of 3243H7 which lowered its SDS-PAGE mobility compared to that of 3243H8. In addition, the concentration of 3243H8 RNA was four-fold higher than that of the epitope-tagged 3243H7 which resulted in significantly higher levels of 3243H8 protein compared to 3243H7. Thus, 3243H8 is tolerated by embryos at levels much higher than those at which 3243H7 produces defects.

## Rescue of normal phenotype by co-expression of a normal connexin

If the developmental defects in embryos expressing 3243H7 were caused by the inhibition of communication, then it should be possible to ‘rescue’ such embryos by co-expression of 3243H7 with another connexin. Rat Cx37 was chosen because it is not inhibited by 3243H7 (see Table 1) and it interacts well

with the endogenous *Xenopus* Cx38 to form communicating channels (Willecke et al., 1991; Bruzzone et al., 1993). Embryos were injected with equivalent amounts of RNAs encoding 3243H7 and rat Cx37 or RNA encoding 3243H7 alone. In two experiments (Table 2), 80% of the 3243H7 injected embryos developed lesions by stage 11, while only 3% of the embryos injected with both 3243H7 and Cx37 RNAs showed developmental defects. In one experiment, one embryo injected with 3243H8 displayed a non-normal phenotype (see Table 2) which appeared qualitatively different than that shown by 3243H7. Embryos injected with Cx37 mRNA alone showed no defects ($n$ = 20, data not shown). A western blot analysis of protein level in these embryos was performed (Fig. 4). 3243H7 protein levels were not affected by the presence of Cx37 (Fig. 4A, compare lanes 1,2). Similarly, Cx37 levels were unaffected by the presence of 3243H7 (Fig. 4B, compare lanes 2,3). These observations suggest that developmental abnormalities produced by 3243H7 were caused by inhibition of communication, not by non-specific or toxic effects of either the RNA or the inhibitor protein itself.

### 3243H7 inhibits communication in the *Xenopus* embryo

The effect of dominant-negative connexin expression on intercellular communication in embryos was analyzed using two different strategies. The first method measured the fraction of embryonic cells capable of communication and is outlined in Fig. 5. Donor cells were prepared from animal caps dye-loaded by incubation with 6-carboxyfluorescein diacetate, which can diffuse across the membrane but is converted intracellularly into the membrane impermeant ester. Recipient cells were prepared from animal caps of embryos whose anterior dorsal blastomeres had been previously co-injected with chimeric connexin RNA and a rhodamine-dextran lineage tracer. Donor and recipient cap cells were dissociated in low $Ca^{2+}$, mixed, then allowed to re-aggregate in $Ca^{2+}$-containing buffer. The reaggregated cells were incubated to permit the formation of intercellular channels permeable to the fluorescein dye. The cells were again dissociated and analyzed by fluorescence microscopy. Recipient cells (rhodamine positive) containing fluorescein were scored as positive for communication. The efficiency of dye transfer using cells from water-injected control embryos did not vary significantly from about 60% over the range of developmental stages tested (stage 6-11; Fig. 6, open bars). Communication involving cells expressing 3243H7 were similar to controls at stage 6 but the efficiency of communication decreased significantly to 40% by stage 7 and to 20% by stage 9, after which no further decreases were observed (Fig. 6, filled bars). Thus, the temporal correlation of inhibition of communication with the appearance of developmental abnormalities (stage 8; see lineage tracing) is very close, suggesting a functional relationship.

These cell-reassociation experiments also permitted observation of the clone of cells injected with 3243H7 over a long time course. The lineage-marked cells, dissociated and cultured in an osmotically balanced medium, were observed to continue cell division and remained viable hours beyond the

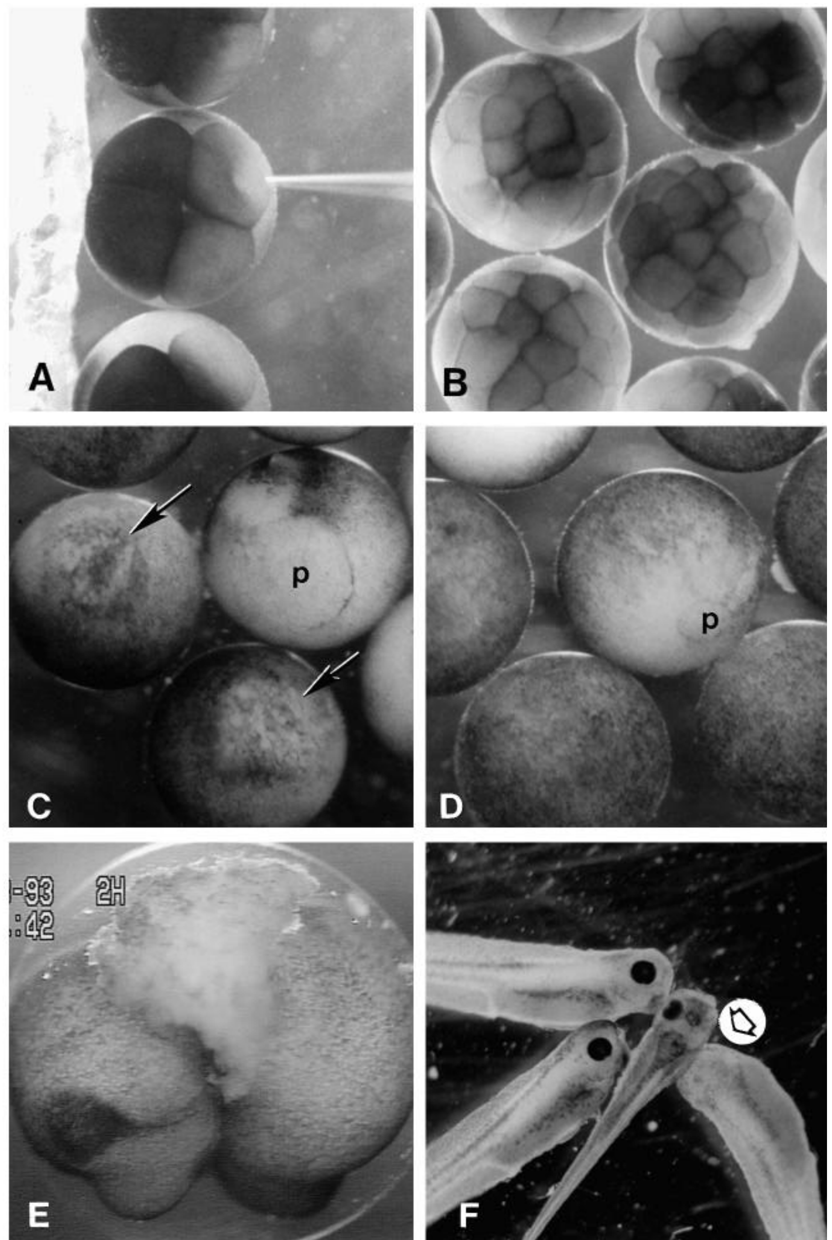


**Fig. 2.** Embryos expressing 3243H7, but not 3243H8, exhibit developmental defects. 8-cell embryos (A) were injected with chimeric connexin RNA in the right anterodorsal blastomere. At early cleavage stages (B), embryos appeared normal. Regional irregularity (arrows) in the ectoderm was evident by mid-gastrulation in embryos expressing 3243H7 (C) but not 3243H8 (D). Just before hatching (E), the extruded cells are still evident. (F) Tadpoles derived from 3243H7-injected embryos (arrow) exhibited loss of anterodorsal structures while those expressing 3243H8 were normal. In C and D, one embryo was rotated during photography to reveal the yolk plug in the blastopore (p).

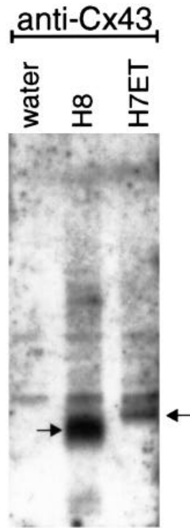


**Fig. 3.** The ability of 3243H7 and the failure of 3243H8 to produce developmental defects is not due to differences in protein levels. Embryos tolerated significantly higher levels of 3243H8 than 3243H7-ET (epitope tagged) without affecting development. Stage 8 embryos that had been injected with water, 3243H8 or 3243H7-ET RNA at the eight-cell stage were Western blotted using an anti-Cx43 antibody recognizing an epitope present in both chimeras. In this experiment, 3243H8 embryos received four times more RNA than 3243H7 embryos, and produced correspondingly higher levels of protein. However, 3243H8 produced no developmental defects. The difference in mobility between the chimeras is due the the presence of an epitope-tag added to 3243H7-ET.

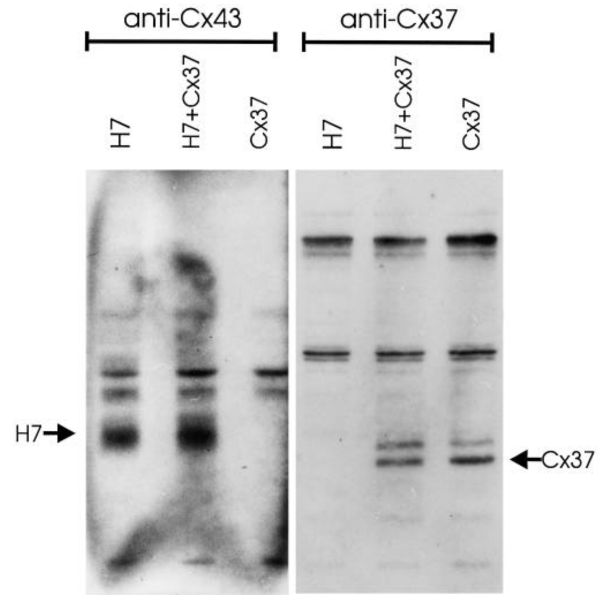


**Fig. 4.** The ability of Cx37 to 'rescue' embryos is not due to depression of inhibitor chimera levels. Embryos injected with Cx37, 3243H7 or Cx37 plus 3243H7 RNA were western blotted with anti-Cx43 (left panel) or anti-Cx37 (right panel) antibodies. Presence of additional RNAs did not affect the levels of protein detected.

time when they would have delaminated from the embryo and lysed in the low ionic strength embryo culture buffers. These observations demonstrate that the progeny of the 3243H7-injected cell are viable and that the delamination of cells cannot be explained by cell death. Longer-term experiments, to determine whether the cells reacquire the ability to form intercellular communication were not attempted.

Direct intracellular dye injections were also used to monitor the ability of individual cells to communicate. In these experiments, 8-cell embryos, co-injected with chimeric connexin RNA and rhodamine-dextran lineage tracer, were allowed to develop to stage 7-8. Microelectrode injections of Lucifer yellow were performed in cells adjacent to the clone of rhodamine/3243H7 RNA injected cells, to measure dye transfer between normal and inhibitor-containing cells. Fig. 7 displays a section from such an embryo where the Lucifer

**Table 2. Rescue of embryos by coinjection of Cx37 with 3243H7**

| mRNA injected at 8-cell stage | 3243H7 | 3243H8 | 3243H7+Cx37 |
|---|---|---|---|
| Experiment 1 | 23/29* | 1†/13 | 1/19 |
| Experiment 2 | 19/23 | 0/14 | 0/16 |
| Totals | 42/52 | 1†/27 | 1/35 |

*Numbers correspond to the no. of embryos expressing delamination phenotype/total no. of embryos, as visually assessed at stage 11.

†The phenotype of this embryo was abnormal but qualitatively different from that produced by 3243H7.

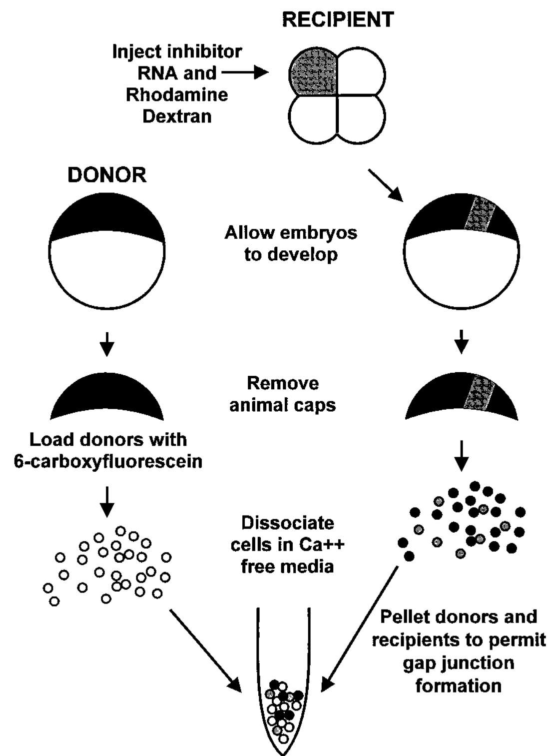


**Fig. 5.** An animal cap re-aggregation assay to monitor intercellular communication. Donor and recipient cells are mixed, allowed to form junctions and transfer dye. The cells are then disaggregated and analyzed by fluorescence microscopy. Cells containing both rhodamine and carboxyfluorescein are scored as positive for communication.

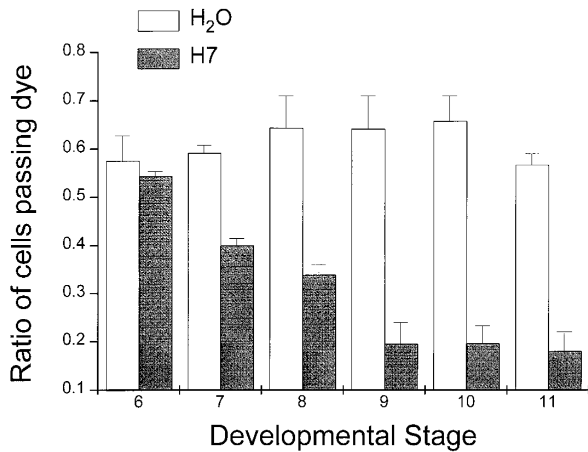


**Fig. 6.** 3243H7 causes inhibition of communication analyzed by the animal cap re-aggregation assay.

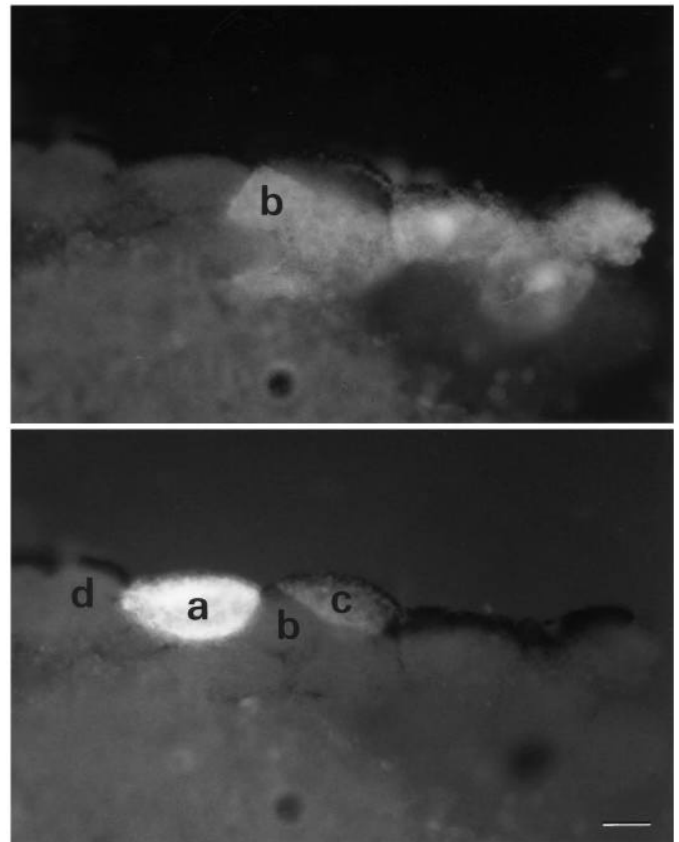


**Fig. 7.** 3243H7 causes inhibition of communication analyzed by dye-injection of Lucifer yellow. The right anterodorsal blastomeres of 8-cell embryos were co-injected with 3243H7 RNA and rhodamine-dextran lineage marker. Lucifer injection was performed at stage 7-8 and embryos were fixed, sectioned and analyzed by fluorescence microscopy for the presence of rhodamine (top panel) and Lucifer(bottom panel). A cell near the clone of marked cells was injected ('a'). Lucifer yellow did not transfer to a neighboring cell containing inhibitor ('b') but did spread to an unmarked neighboring cell ('c'). Dye transfer to unmarked cells was sometimes not observed ('d'). Transfer to a marked cell was never observed. Bar = 43 μm.

yellow injected cell (Fig. 7, bottom panel, cell 'a') was outside the clone of rhodamine/3243H7 containing cells (compare top and bottom panels). Dye did not diffuse into an adjacent rhodamine-labeled cell (Fig. 7, top panel, cell 'b') but freely diffused into a non-inhibited neighbor (cell 'c'). Lucifer yellow transfer to inhibited cells was never observed in 10 separate experiments, indicating that expression of 3243H7 inhibited the ability of cells to communicate *in situ* in the developing embryo. Dye transfer was not uniform in embryos at this developmental stage. For example, cell 'd' (Fig. 7) is immediately adjacent to the injected cell, but did not receive dye. This suggests a regionality or compartmentalization of communication in the late blastula. Together, the results of the reaggregation and direct dye-injection assays indicated that 3243H7 caused significant inhibition of communication in the *Xenopus* embryo.

### Lineage tracing

A lineage tracing study was performed to determine the fates of communication-inhibited cells. 8-cell-stage embryos were co-injected in the right anterodorsal blastomere with 3243H7 RNA and a colloidal gold-BSA conjugate (Niehrs and DeRobertis, 1991). Embryos were allowed to develop to various stages then fixed, embedded and serially sectioned. In control embryos at stage 8, the surface ectoderm overlying the blastocoel was organized into superficial epithelial (occluding) and deep nonepithelial (sensorial) layers (Keller, 1991; Fig. 8A). Serial sectioning of the embryos revealed that all progeny of the originally injected cell remained in an intact cluster. Fig. 8C shows a grazing section of an embryo, which missed the blastocoel, where this clustering is evident. This organization was markedly disturbed in embryos of the same stage expressing 3243H7. In some regions containing cells expressing the inhibitor, there appeared to be only one layer of cells (Fig. 8B), while in other areas, individual blastomeres could be observed delaminating from the surface (Fig. 8D). By stage 10, cells were often also observed in the blastocoel (Fig. 8E), in addition to the delamination and thinning lesions seen earlier. By stage 12.5, the latest time analyzed, large numbers of marked cells were extruded from the 3243H7 RNA injected embryos, although a significant number of marked cells could be still detected (Fig. 8F).

## DISCUSSION

We have produced a chimeric connexin that functions as a dominant-negative inhibitor of intercellular communication in developing *Xenopus* embryos. Inhibition of communication in the progeny of 8-cell-stage anterodorsal blastomeres leads to regional delamination of surface ectoderm in 70-80% of injected embryos. Since identification of the dorsal cells using pigmentation is only 80% accurate, one explanation for the failure to observe 100% defects would be mistargeting of injections to ventral blastomeres (Moody, 1987). Lineage tracing indicates that most of the inhibited cells are absorbed or extruded by the embryo but some persist and presumably contribute to adult structures. Injections did not appear to interfere with or alter the timing of cell divisions. Mild to severe pat-

terning defects resulted from the loss of derived structures. Two lines of evidence support the idea that suppression of intercellular communication was the mechanism responsible for the developmental defects observed. First, the time course of the inhibition of communication in the embryo was well-correlated with the appearance of the defects. Second, the defects could be rescued by the expression of a connexin that was not subject to the action of the dominant negative inhibitor.

A dominant-negative connexin mutation could interfere with several aspects of intercellular channel formation. Assuming that 3243H7 oligomerizes with a compatible connexin to form heteromeric connexons and that oligomerization of subunit connexins occurs in the trans-Golgi (Musil and Goodenough, 1993), 3243H7 could inhibit the intracellular movement of connexons from the trans-Golgi compartment to the plasma membrane. Immunofluorescence localization of 3243H7 in transient transfections of mammalian tissue culture cells (data not shown) revealed little or no cell surface labeling. Thus, it is possible that 3243H7 causes endogenous connexins to be retained in an intracellular membrane compartment. Alternatively, the presence of 3243H7 could prevent either the extracellular alignment of connexons in adjacent cells to form a

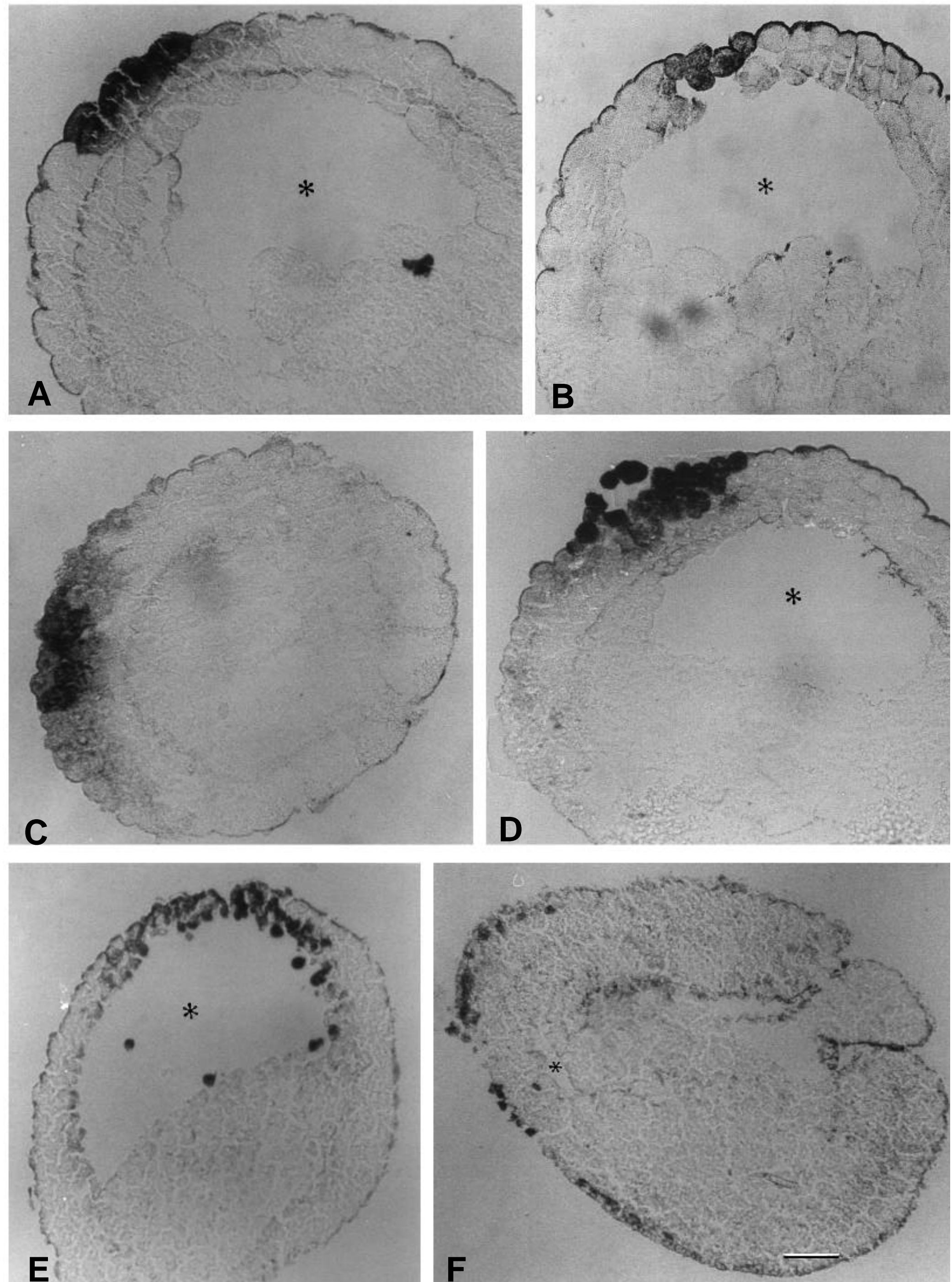


**Fig. 8.** Lineage tracing of clones of cells derived from injected 8-cell blastomere. The right anterodorsal blastomere was injected with either water (control) or 3243H7 mRNA together will the lineage marker of Niehrs and DeRobertis (1991). Embryos were fixed, embedded in paraffin and sectioned. (A) Control embryo, stage 8. All marked blastomeres are tightly clustered with no obvious intercellular spaces. (B) 3243H7-injected, stage 8. Some of the marked clone of cells show loss of cell-cell contact and the sensorial layer of the ectoderm is missing. (C) Control embryo, stage 8. The marked clone of cells appear clustered, even at the edge of the clone. (D) 3243H7 injected, stage 8. The clustering of the marked blastomeres is less tight and individual cells can be seen delaminating from the embryo surface. (E) 3243H7-injected, stage 10. Cells of the marked clone now appear widely scattered; some have fallen to the floor of the blastocoel. (F) 3243H7-injected, stage 12.5. While many members of the marked clone of cells have been lost, a variable number remain scattered in different embryos. Bar = 100 μm

complete intercellular channel or the gating of the completed channel into an open configuration. However, the molecular mechanism by which 3243H7 inhibits communication is not clear at this time.

Warner et al. (1984) used anti-connexin antibody injections to inhibit communication during *Xenopus* embryogenesis. Effective communication blockade was evident by the 32- to 64-cell stage, much earlier than our first observation of reduced communication, at stage 7. In addition, Warner et al. (1984) were able to completely block intercellular communication, while our assay demonstrated 20% of progeny cells still capable of intercellular communication. Since Warner et al. (1984) did not measure intercellular communication beyond the 32-cell stage, it is not clear whether the block to intercellular communication overlapped with the developmental stages studied here. In addition, the delamination of ectodermal cells observed in our study was not reported by Warner et al. (1984) which should have been evident in the histological sections provided. Thus, inhibition of communication at these two developmental stages has very different effects, making it very difficult to compare these two studies.

In typical experiments the delamination defect is first evident at stage 7, presumably because inhibitor concentrations do not reach high enough levels until this time. Although detailed dose-response experiments have not been performed, injection of equivalent amounts of RNA at the 1-, 2-, or 4-cell stages did not cause defects to appear significantly earlier, although the size of the effected clone was appropriately larger (data not shown). While the experiments of Warner et al. (1984) demonstrate that communication is a critical factor for development at earlier stages, the earliest stage at which a requirement for communication exists cannot be defined using the RNA injections used in the present study. Host-transfer techniques could be used to determine if 3243H7 has an effect on maternal adhesion systems (Heasman et al., 1994).

The phenotype produced by inhibition of communication in progeny of anterodorsal blastomeres most resembles those observed in studies where cadherin-based adhesion systems were experimentally perturbed (Heasman et al., 1994; Detrick et al., 1990; Fujimori et al., 1990; Kintner, 1992; Levine et al., 1994). The cadherins are the most well-characterized class of adhesion molecules present during the stages of development where 3243H7 effects are evident (Choi et al., 1990; Angres et al., 1991; Herzberg et al., 1991; Choi and Gumbiner, 1989; Detrick et al., 1990). Embryos ectopically expressing large amounts of N-cadherin show perturbations in tissue histogenesis (Fujimori et al., 1990) or may develop lesions in the ectoderm during gastrulation similar in appearance to those produced by 3243H7 (Detrick et al., 1990). In the latter study, close cellular associations are lost, presumably because high levels of exogenous N-cadherin competitively inhibit intracellular interactions of other cadherins. In keeping with this hypothesis, expression of N-cadherin with a truncated extracellular domain also caused a loss of ectodermal integrity (Kintner, 1992). Cells from the sensorial layer dissociated and migrated into the blastocoel before gastrulation, while disturbances in the occluding layer were detected by mid-gastrulation.

Heasman et al. (1994) used the host-transfer strategy combined with antisense DNA oligonucleotides to deplete blastula stage embryos of EP- (also known as C-) cadherin. These embryos appear to have normal surface cells, but show loss of adhesion between inner cells, with a concomitant loss of the blastocoel. In embryos expressing 3243H7, we observed no loss of blastocoel structure similar to that described by Heasman et al. (1994) indicating either that insufficient gap junctional blockade had developed at these earlier stages, or that the C-cadherin adhesion system does not require gap junctional intercellular communication for stabilization. More recently, Levine et al. (1994) expressed an exogenous E-cadherin with a truncated cytoplasmic domain in *Xenopus* embryos at the 2-cell stage. In these embryos, lesions similar to those reported here and by Kintner (1992) were described, but the cellular dissociation observed at gastrulation involved primarily the occluding and not the sensorial ectodermal layer. Thus E-cadherin functions in maintaining adhesion between cells whose positions remain invariant, characteristic of epiboly; C-cadherin is found between cells that need to shift positions as during convergence and extension movements. Our results indicate that the stable, E-cadherin-type associations are more sensitive to the effects of 3243H7 blockade of gap junctional intercellular communication, but that both systems may be affected, similar to the effects caused by the extracellularly truncated N-cadherin construct of Kintner (1992). The relationship between gap junctional communication and cell adhesion is likely to be complex and involve multiple regulatory pathways. In this regard, we were unable to rescue 3243H7 embryos by co-injection of E-cadherin mRNA (the generous gift of Dr Barry Gumbiner, data not shown).

These observations are consistent with a model in which cadherin-based intercellular adhesion and intercellular communication via gap junctions provide mutual homeostatic feedback signals. Previous studies indicate that cadherin-based adhesion contributes to the establishment of gap junctions (Mège et al., 1988; Musil et al., 1990; Meyer et al., 1992). Conversely, other published studies suggest that signal transduction through gap junctions may modulate adhesion. Lee et al. (1987) showed that microinjection of anti-Cx32 antibodies into one blastomere at the compacted 8-cell-stage mouse embryo results in a blockade in intercellular communication and an extrusion of the antibody-containing cells, analogous to the delamination and extrusion described here, but on a more rapid time scale, consonant with the direct effects of antibody binding. A blastomere injected with the same antibody at the 2-cell stage continues to divide normally, but the progeny fail to compact at the 8-cell stage. The close relationship between gap junctional communication and compaction is further supported by studies of the DDK inbred mouse strain (Buehr et al., 1987) and by Cx32 antisense RNA injections into preimplantation embryos (Bevilacqua et al., 1989). In keeping with the hypothesis that communication and adhesion are mutually interdependent, compaction also requires not only communication but also functional E-cadherin (Johnson et al., 1986), since it can be inhibited with anti-E-cadherin antibodies. Similarly, treatment of Novikoff hepatoma cells with anti-connexin antibodies directed at extracellular domains are equally as effective as antibodies specific for A-CAM (N-cadherin) in blocking the assembly of adherens junctions (Meyer et al., 1993). Taken together with our results, these data indicate that intercellular adhesion may require signaling through gap junctional pathways for long-term homeostasis or

programmed developmental switching between different adhesion systems.

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