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Translation and Functional Expression of Cell-Cell Channel mRNA in *Xenopus* Oocytes

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Summary. mRNA from estrogen-stimulated rat myometrium, a tissue known to upregulate cell-cell channels in response to this hormone, was microinjected into *Xenopus laevis* oocytes. The oocytes had been freed from covering layers of follicle cells and vitelline to allow direct cell membrane interactions when paired. About 4 hours after the mRNA injection, paired oocytes become electrically coupled. This coupling was due to the presence of typical cell-cell channels characterized by size-limited intercellular tracer flux, the presence of gap junctions at the oocyte-oocyte interface, and the reversible uncoupling that occurred in the presence of carbon dioxide. The induction of new cell-cell channels in the oocyte membrane was observed against a zero background or a low level of endogenous coupling, depending on the maturation stage of the oocytes. The time course of development of cell-cell coupling after the microinjection of mRNA was determined. The mRNA capable of inducing cell-cell coupling was confined to an intermediate size class when fractionated on a sucrose gradient.

Key Words cell-cell channels · mRNA · size fractionation · *in vivo* translation · *Xenopus* oocytes

Introduction

Advances in recombinant DNA technology have made it possible to investigate the structure of membrane channel proteins. Knowing the partial amino acid sequences of the acetylcholine receptor and the sodium channel proteins, Numa and coworkers (Mishina et al., 1984; Noda et al., 1984) were able to isolate cDNA clones for these proteins using synthetic hybridization probes. The mRNA transcribed from such cDNA clones was then injected into *Xenopus* oocytes, and the properties of the expressed proteins could be studied by electrophysiological methods. We have used a similar approach, although in reverse (Dahl, Azarnia & Werner, 1980, 1981). Our approach was designed to identify and characterize a membrane protein without ever having isolated it. To accomplish this, total mRNA from a cell that expresses this membrane protein was introduced into another cell that is normally deficient in the functional expression of this protein. The mRNA-directed expression of the unidentified protein in the recipient cell was then assayed by its function. We are using this functional assay for the purification of the mRNA of the protein via molecular cloning.

We were interested in studying the cell-cell channel (gap junction) protein. There has been a consensus that the cell-cell channel consists of a hexamer of 27,000-dalton subunits (Caspar et al., 1977; Makowski et al., 1977; Henderson, Eibl & Weber, 1979; Hertzberg & Gilula, 1979; Finbow et al., 1980; Unwin & Zampighi, 1980). This consensus was based on an isolation procedure that had to rely solely on morphological appearance of gap junctions in membrane preparations. Such a procedure, however, allows only minimum estimates of the number of components in the functional channel and of the molecular weight of the protein. This limitation cannot be dismissed in light of the fact that the morphology of the junction remains unchanged even after intentional proteolysis (Goodenough, 1976). Because of the obvious drawbacks of such an approach, we attempted to identify the cell-cell channel proteins by a different experimental approach. We isolated mRNA from a tissue actively involved in junction formation, injected it into a junction-deficient cell, and assayed functionally for the appearance of cell-cell channels in the cell's membrane. As source of mRNA we chose estrogen-induced rat myometrium, which had previously been shown to upregulate gap junctions (Dahl & Berger, 1978; Garfield, Kanaan & Daniel, 1980). The mRNA was then introduced via liposomes into junction-deficient Cl-1D cells (a mouse L-cell derivative). In response to the mRNA infusion, the Cl-1D cells became electrically coupled (Dahl et al., 1980, 1981).

While this approach worked, it soon became apparent that the liposome-mediated delivery of

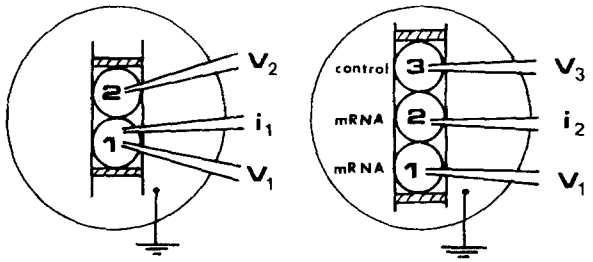

Fig. 1. Arrangement of oocytes in pairs (left) and triplets (right). The microelectrodes are used to inject current, *i*, or record the cell membrane potential, *E*, and its electrotonic displacement, *V*. For details *see* text

mRNA into a cell was rather inefficient. More serious, however, was the fact that the procedure was prohibitively expensive. In this publication, we describe an alternative assay that involves the microinjection of mRNA into *Xenopus* oocytes.[1] The results obtained with this new assay confirm our previous conclusions and provide evidence that typical cell-cell channels can be expressed in the oocyte membrane. The expressed channels can be reversibly closed by established uncoupling procedures.

We also report on the partial purification of cell-cell channel mRNA by sucrose gradient sedimentation. Channel-producing mRNA is confined to a certain size class.

Materials and Methods

Isolation of mRNA

The preparation of animals and the isolation of mRNA have been described previously (Dahl et al., 1980, 1981). Virginal Sprague Dawley rats are injected intramuscularly for 2 consecutive days with Delestrogen. On the third day, the rats are sacrified, the uteri removed, and the myometrica mechanically separated from the endometrium.

Myometria suspended in guanidinium thiocyanate (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% Sarkosyl, 5% beta-mercaptoethanol) are homogenized in an Ultra Turrax® homogenizer, the mixture centrifuged, and the supernatant extracted twice with hot phenol (Chirgwin et al., 1979). After precipitation with ethanol, the total RNA is sedimented through a CsCl density gradient. mRNA is further purified by chromatography on oligo-dT cellulose.

[1] While we were developing the oocyte assay Miledi's group reported the use of oocytes for the translation and functional assay of messengers for voltage-gated membrane channels and for the nicotinic acetylcholine receptor (Miledi, Parker & Sumikawa, 1982; Gundersen, Miledi & Parker, 1984).

Preparation of Oocytes

Xenopus laevis frogs were obtained from Nasco or Xenopus I. Ovaries were removed and stage VI oocytes (Dumont, 1972) collected. The layers covering the oocytes (collagen, follicle cells, and vitelline membrane) are removed with fine forceps under a dissecting microscope. Oocytes are kept in Barth saline (88 mM NaCl, 1 mM KCl, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 2.4 mM $NaHCO_3$, 10 mM HEPES, pH 7.4, 0.1% glucose, 10 mg/liter benzyl penicillin, 10 mg/liter streptomycin sulfate). Stripped oocytes are mounted in pairs or triplets in closely fitting Teflon grooves (*see* Figs. 1 and 10).

Microinjection of mRNA

mRNA dissolved in distilled water (1 mg/ml) is taken up into microcapillaries and injected into oocytes by application of pressure. About 30 ng of mRNA is injected per oocyte.

Recording of Electrical Coupling

For the measurement of electrical cell-cell coupling we used the two types of arrangement shown in Fig. 1. In pairs, both oocytes are injected with mRNA. Control oocytes are not injected at all or are sham-injected. In the triplet formation, two neighboring oocytes (*1* and *2*) are injected with mRNA and the third oocyte serves as control (noninjected or sham-injected). For coupling measurements in oocyte pairs, one oocyte (*1*) is impaled with both a current electrode (delivering hyperpolarizing square wave current pulses of 20 nA and 600 msec) and a voltage recording electrode; the second oocyte (*2*) is impaled with a voltage recording electrode. The ratio of the displacements of the membrane potentials in oocyte *2* and *1*, V_2/V_1, gives the coupling ratio; at low values it is proportional to the number of cell-cell channels (Socolar & Loewenstein, 1979). In triplets, oocytes *1* (mRNA-injected) and *3* (control) are impaled with voltage recording electrodes, and the current electrode is inserted into oocyte *2* (mRNA-injected). Displacements of the membrane potentials in oocytes *1* or *3* indicate electrical coupling to oocyte *2*, and the amplitude of the displacement is a relative measure of the degree of coupling. Unless stated otherwise, measurements were taken 12–18 hr after the injection of mRNA.

Tracer Transfer Studies

Carrier-free ^{35}S-sulfate is microinjected into the center oocyte of a triplet formation 12 hr after the injection of mRNA (*see* Table 1). One hour later, the oocytes are separated mechanically, washed repeatedly, transferred into scintillation vials, and lysed. Radioactivity is determined in a liquid scintillation counter. Identical procedures were used for the injection of ^{125}I-labeled transferrin.

Electron Microscopy

Oocytes were fixed at room temperature in a solution of 2% glutaraldehyde buffered with Na-cacodylate (pH 7.4). After 1 hr the fixing solution was replaced by a fixative containing 30% glycerol. Interfaces of oocyte pairs were dissected, mounted on specimen carriers, and frozen in melting Freon 22. Fracturing

and replication was performed according to standard techniques in a Balzers 301 freeze-fracture device. Platinum-carbon replicas were examined in a Phillips electron microscope EM 300.

Fractionation of mRNA

200 μg of mRNA were precipitated with ethanol, and the precipitate was dissolved in 70% formamide. After heating to 70°C for 2 min the mRNA solution was layered onto a denaturing sucrose gradient (70% formamide, 4.6 to 22% sucrose) and sedimented at 25°C for 24 hr at 35,000 rpm in a Spinco SW-41 rotor (Suzuki, Gage & Brown, 1972). Nine fractions were collected and individually precipitated with ethanol.

Results

Preparation of Oocytes

Xenopus oocytes are covered with three layers, an outermost layer of connective tissue, a single layer of follicle cells, and an inner layer of vitelline, a protein intimately attached to the oocyte plasma membrane (Fig. 2). Microvilli of both oocyte and follicle cell penetrate the vitelline layer and occasionally make contact. At such contact sites, two types of membrane junction can be found, gap junctions and tight junctions (Fig. 2). These junctions can be seen in all oocytes independent of their stage of maturation. We have looked at stages 4 through 6. Although there may be quantitative differences in the occurrence of junctions, we were reluctant to initiate a morphometric analysis because the oocyte membrane is unfavorable for such a task (*see* Fig. 2*a*).

In order to achieve contact between oocyte cell membranes, all surface layers, including the vitelline layer, are removed mechanically (Fig. 3). The resulting "stripped" oocytes still exhibit junctional structures on their surface. These junctions probably represent oocyte-follicle cell junctions where the complete junction was torn out of the follicle cell membrane. Such a mechanism has been shown to occur in various cell separation procedures. In dissociated culture cells (Preus, Johnson & Sheridan, 1981), collagenase-treated heart cells (G. Isenberg and G. Dahl, *unpublished results*), and in dissociated *Xenopus* embryo cells (B. Rose and G. Dahl, *unpublished observations*), junctions were seen between a single cell and a small membrane vesicle. It is conceivable that such junctions can be re-used by fusion of the vesicle with the cell membrane of an oocyte brought into contact with it. Since such a mechanism of junction formation would give us false-positive results, we keep the stripped oocytes as single cells for at least 4 hr. After that time, most of the oocytes did not exhibit junctional structures on their plasma membranes. Instead, junctional structures could be seen associated with putative endocytotic vesicles (Fig. 4).

Electrical Coupling between Oocytes

Stripped oocytes have a membrane potential of -38.4 ± 1.0 mV ($n = 77$). The mean value for the membrane potential of mRNA-injected oocytes was slightly higher: -40.3 ± 1.0 mV ($n = 47$). The input resistance of single oocytes is in the order of 0.5 Ω. Oocytes retain these properties for at least 2 days after dissection. For measurement of electrical coupling between oocytes we initially used oocyte triplets. Two adjacent cells were injected with mRNA, the third served as control (Fig. 5). The incidence of electrical coupling between mRNA-injected pairs was found to be 21/29, and 10/29 between control cells and their mRNA-injected neighbors. Control cells were coupled to the mRNA-injected oocyte only in those triplets in which the two mRNA-injected oocytes were also coupled. The extent of coupling was always greater between two mRNA-injected cells than between control and mRNA-injected cells, in most cases by a factor of 3 or more (Table 1). Since coupling ratio is a nonlinear function of the number of channels, we measured junctional conductance in a few triplets using a double voltage-clamp technique (in collaboration with B. Rose, *unpublished results*). These more accurate measurements of the number of channels showed that the induction of channels is larger than coupling ratios can reveal.

In order to evaluate the capacity of control oocytes to establish electrical coupling we mounted several noninjected oocytes in pairs. Surprisingly, these pairs showed absolutely no electrical coupling even though an oocyte from the same donor exhibited electrical coupling when used as control in a triplet formation. The results reported so far were all obtained with oocytes from a single shipment of frogs. Subsequently, we learned that oocytes by themselves are capable of forming cell-cell junctions depending on what appears to be their maturation state (Table 2). Oocytes from some frogs were found to be completely free of endogenous cell-cell channels, while oocytes from other donors exhibited low cell-cell coupling when tested in pairs. In batches without background coupling, mRNA was found to induce cell-cell coupling with a mean coupling ratio of about 0.3. In batches exhibiting endogenous channels, mRNA induced cell-cell channels by almost the same increment, i.e., the coupling ratio increased from 0.1 to 0.4.

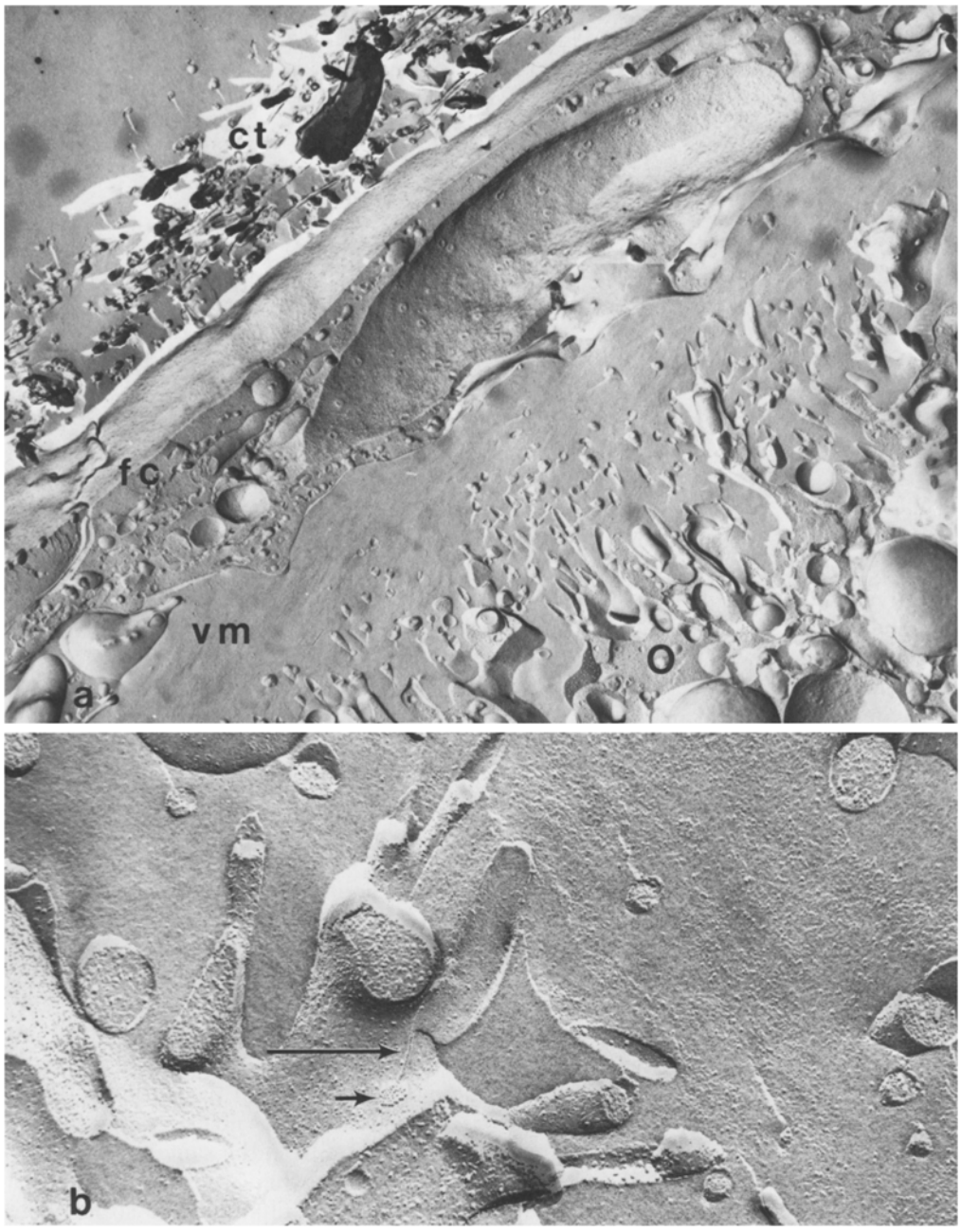

Fig. 2. Morphology of oocytes. (*a*) Intact oocytes are covered by connective tissue (*ct*), a layer of follicle cells (*fc*), and the vitelline membrane (*vm*). The plasma membrane of the oocyte (*O*) exhibits a rough surface provided by microvilli that interdigitate through the vitelline membrane with villus projections from the follice cells. (*b*) At sites of contact between follicle cell and oocyte membranes, gap junctions (short arrow) and tight junctions (long arrow) can be observed. Magnifications: (*a*) ×13,000; (*b*) ×49,500

The observation that oocytes by themselves are capable of forming cell-cell channels is not surprising in light of the fact that they normally establish junctions with follicle cells. However, the number of copies of hemichannels being expressed in an oocyte membrane is probably so low that the chance to establish functional channels between two neighboring oocytes is relatively small. In contrast, when a control oocyte faces the membrane of an mRNA-injected oocyte, where hemichannels are presumably much more prevalent, the formation of functional channels is more favorable. As a consequence of these considerations we continued our studies using oocyte pairs, rather than triplets, thus

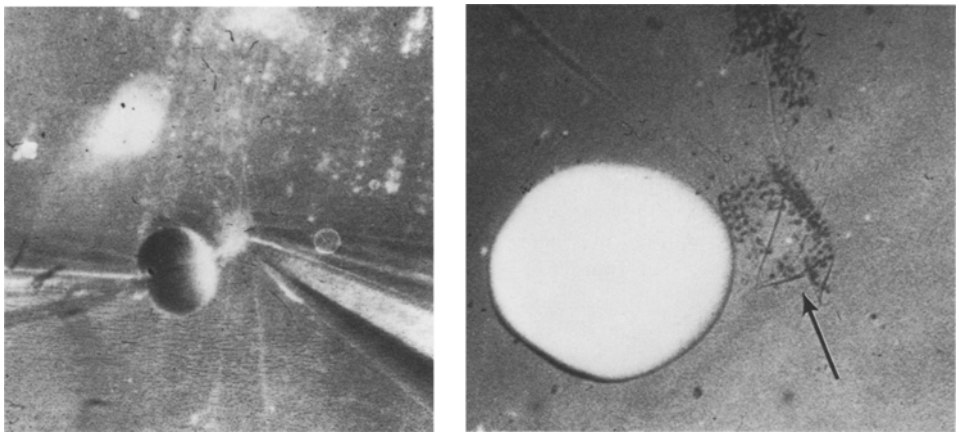

Fig. 3. Dissection of oocytes. The vitelline membrane is removed mechanically by forceps (left) and can be seen separated from the oocyte on the right (arrow). The dark spots represent a few remaining follicle cells

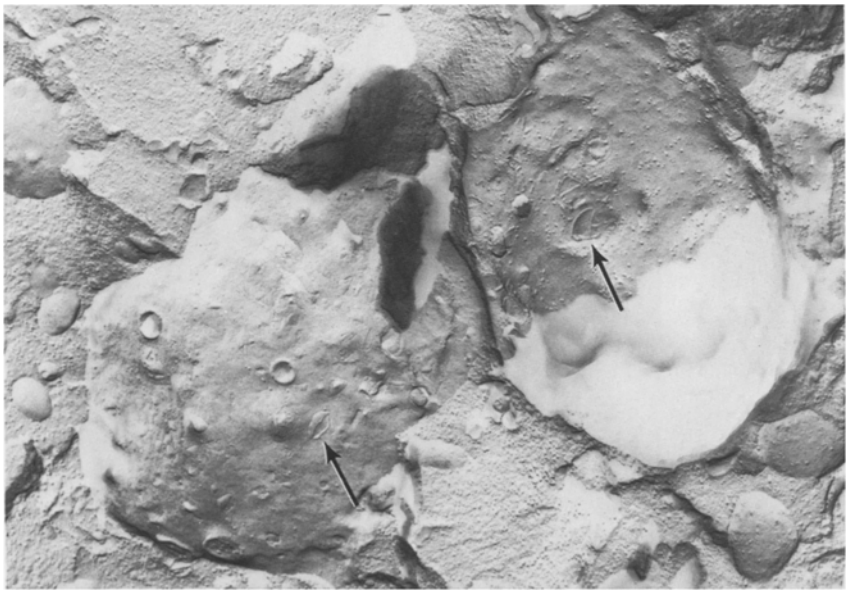

Fig. 4. Junctional structures on membranes within the oocyte. Bimembranous vesicles exhibiting tight junctional strands (arrow) are found several microns away from the plasma membrane. They presumably represent endocytotic vesicles. Magnification: ×38,000

avoiding the possible recruitment of low abundancy hemichannels by a cell expressing high numbers of hemichannels.

Transfer of ^{35}S-Sulfate between Oocytes

One of the characteristics of cell-cell channels is that they allow transfer of molecules from one cell to another. Such transfer occurs with molecules up to a molecular weight of about 1000 daltons (Loewenstein, 1981). Tracer molecules most commonly used in monitoring the junctional transfer of molecules are fluorescent dyes. Such tracers are difficult to use in *Xenopus* oocytes as these cells exhibit autofluorescence over a wide spectral range. We, therefore, used ^{35}S-sulfate as a tracer molecule to demonstrate cell-to-cell transfer. We used triplets of oocytes as described above. Twelve hours after mRNA injection, the central cell of a triplet was injected with ^{35}S-sulfate. After incubation for 1 hr, the cells were separated mechanically, washed, lysed, and their ^{35}S content was determined in a liquid scintillation counter. The results are included in Table 1. Significant transfer of label was only observed to the mRNA-injected oocyte. The radioactivity of the control oocyte was only slightly above background.

In parallel experiments we injected into the central oocyte ^{125}I-labeled transferrin, a molecule too large to pass through cell-cell channels. Neither mRNA-injected neighbor nor control cell showed any increase of radioactivity above background. This result precludes the presence of cytoplasmic continuity, other than provided by cell-cell channels, between neighboring oocytes.

Table 1.

	a) Electrical coupling between oocytes in triplets	
	Observed change in membrane potential in mV	Coupling incidence
mRNA-injected cell (1)	2.7 ± 0.6	21/29
Control cell (3)	0.7 ± 0.2	10/29
	b) Transfer of ^{35}S-sulfate (cpm)	c) Transfer of ^{125}I-transferrin (cpm)
mRNA-injected cell (1)	252 ± 71 (n = 50)	48 ± 7 (n = 13)
Tracer-injected cell (2)	94,923 ± 16,820	2,447 ± 395
Control cell (3)	71 ± 13	51 ± 13

Experimental data are expressed as means ±SE. Background counts averaging 29 cpm were not subtracted.
^{35}S-counts in line (1) are different from ^{35}S-counts in line (3) with a confidence level $P < 0.01 > 0.005$. Five of the oocyte triplets were scored electrophysiologically before their radioactivity was determined. ^{35}S-sulfate transfer and electrical coupling were found to be correlated. Noncoupling cells showed no tracer transfer

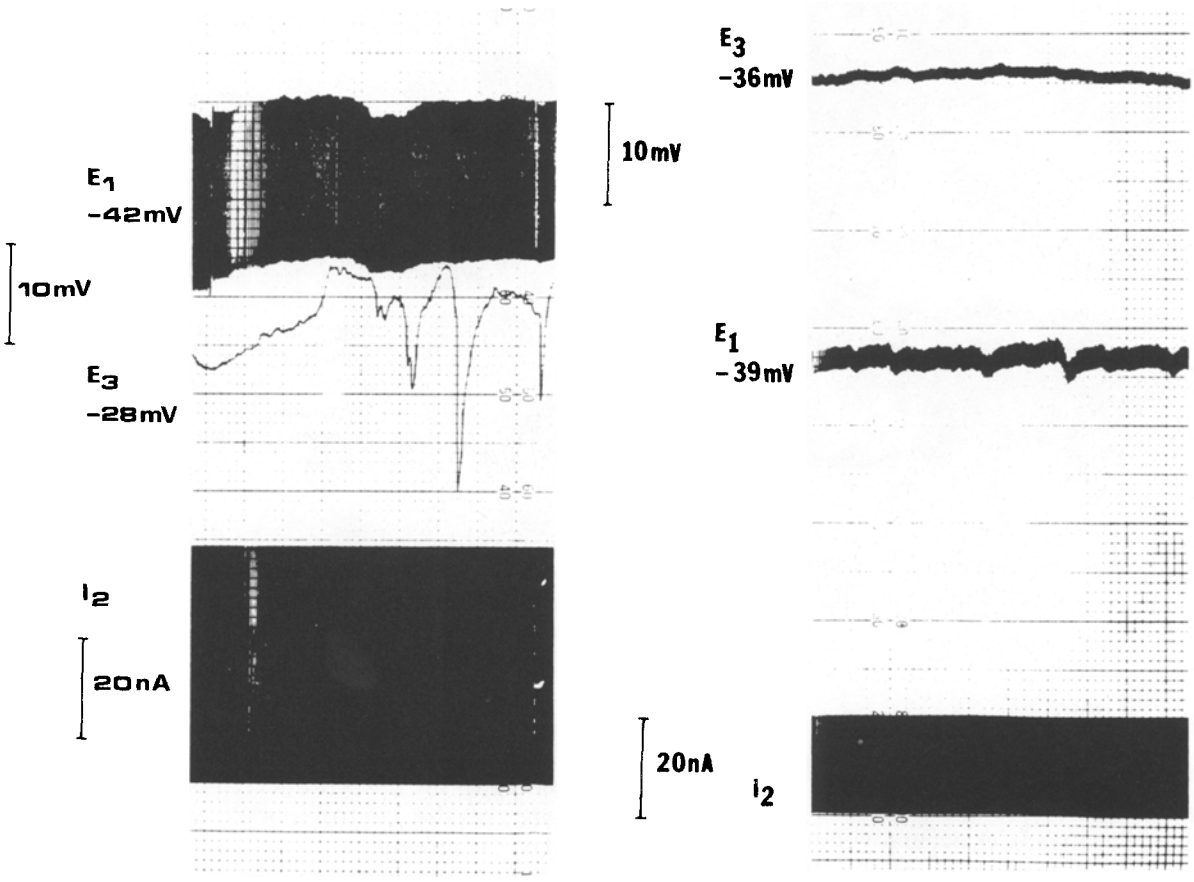

Fig. 5. Electrical coupling in oocyte triplets. The mRNA-injected center cell receives current pulses (I_2); membrane potential is recorded from the mRNA-injected neighbor cell (E_1) and from the control neighbor (E_3). (left) The membrane potential of the mRNA-injected neighbor cell is displaced (V_1) by current pulses applied to the mRNA-injected center cell (I_2), whereas the membrane potential of the control cell remains unaffected ($V_3 = 0$). E_3 shows spontaneous depolarizations, which are observed in oocytes occasionally over short time periods. (right) Electrical coupling is present not only between the two mRNA-injected cells (V_1) but also between the mRNA-injected center cell and the control cell (V_3). The displacement of the membrane potential, however, is larger in oocyte 1 than in oocyte 3

Table 2. Coupling between oocytes in pairs

	mRNA-injected oocytes		Control oocytes	
	Coupling ratio	Coupling incidence	Coupling ratio	Coupling incidence
No background	0.30 ± 0.04	34/ 52	0	0/ 53
With background	0.43 ± 0.04	53/ 60	0.13 ± 0.02	33/ 65
Total	0.37 ± 0.03	87/112	0.07 ± 0.02	33/118

Experimental data are expressed as means ±SE. The table does not include data from early experiments where coupling was measured qualitatively only (two electrodes). Inclusion of these data would increase the number of pairs that did not show any background by 50.

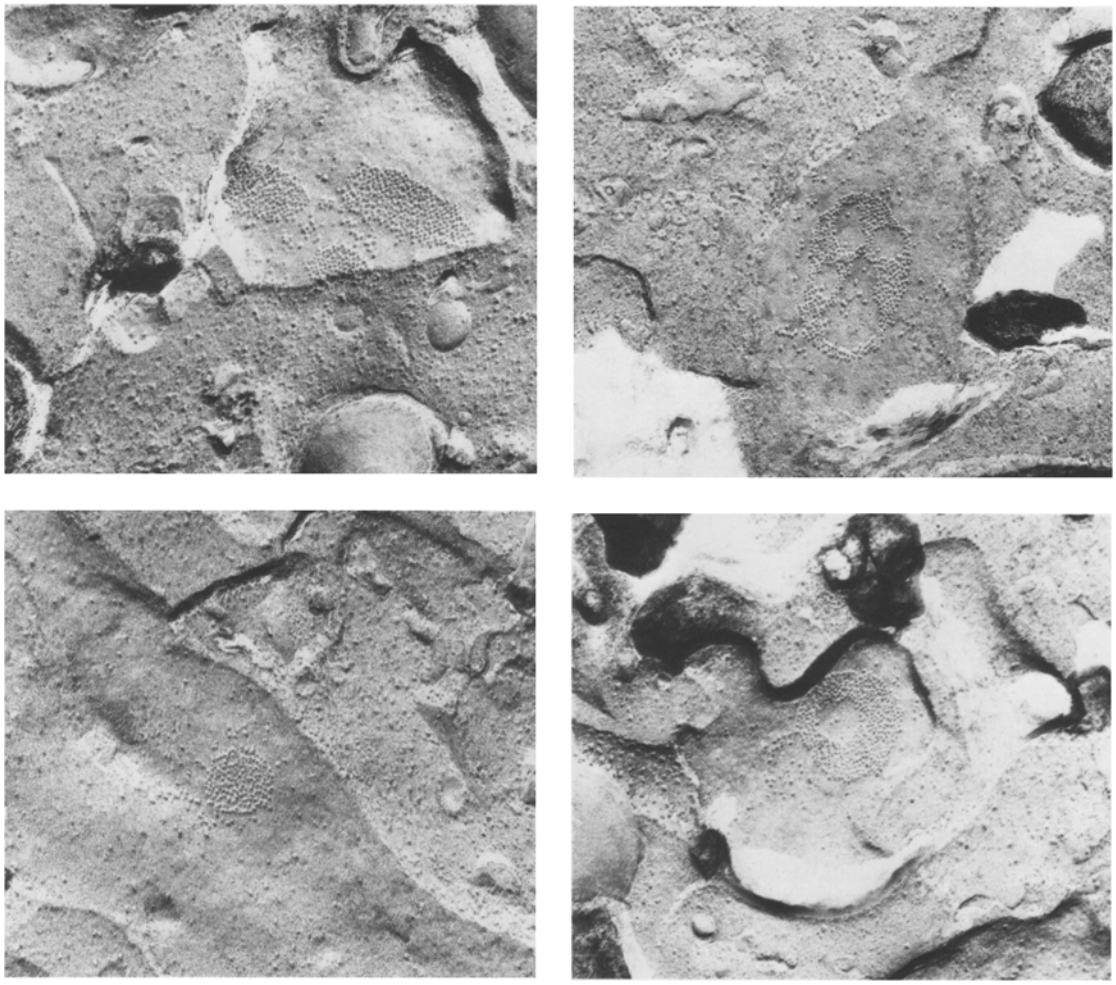

Fig. 6. Electron micrographs of an mRNA-injected oocyte pair. The fracture plane exposed a large portion of the interface, and several more gap junctional plaques than shown here were observed. Magnification: ×56,000

Visualization of Gap Junctions

Another characteristic of cells communicating by cell-cell channels is the presence of clusters of particles (gap junctions) in freeze-fractured membranes at membrane-membrane contact areas. These particles are generally considered to represent the cell-cell channels. We were successful in freeze-fracturing the cell-cell contact area of three mRNA-injected oocyte pairs. We found typical gap junctions in all three (Figs. 6 and 7). The interface of the one oocyte pair with endogenous coupling that

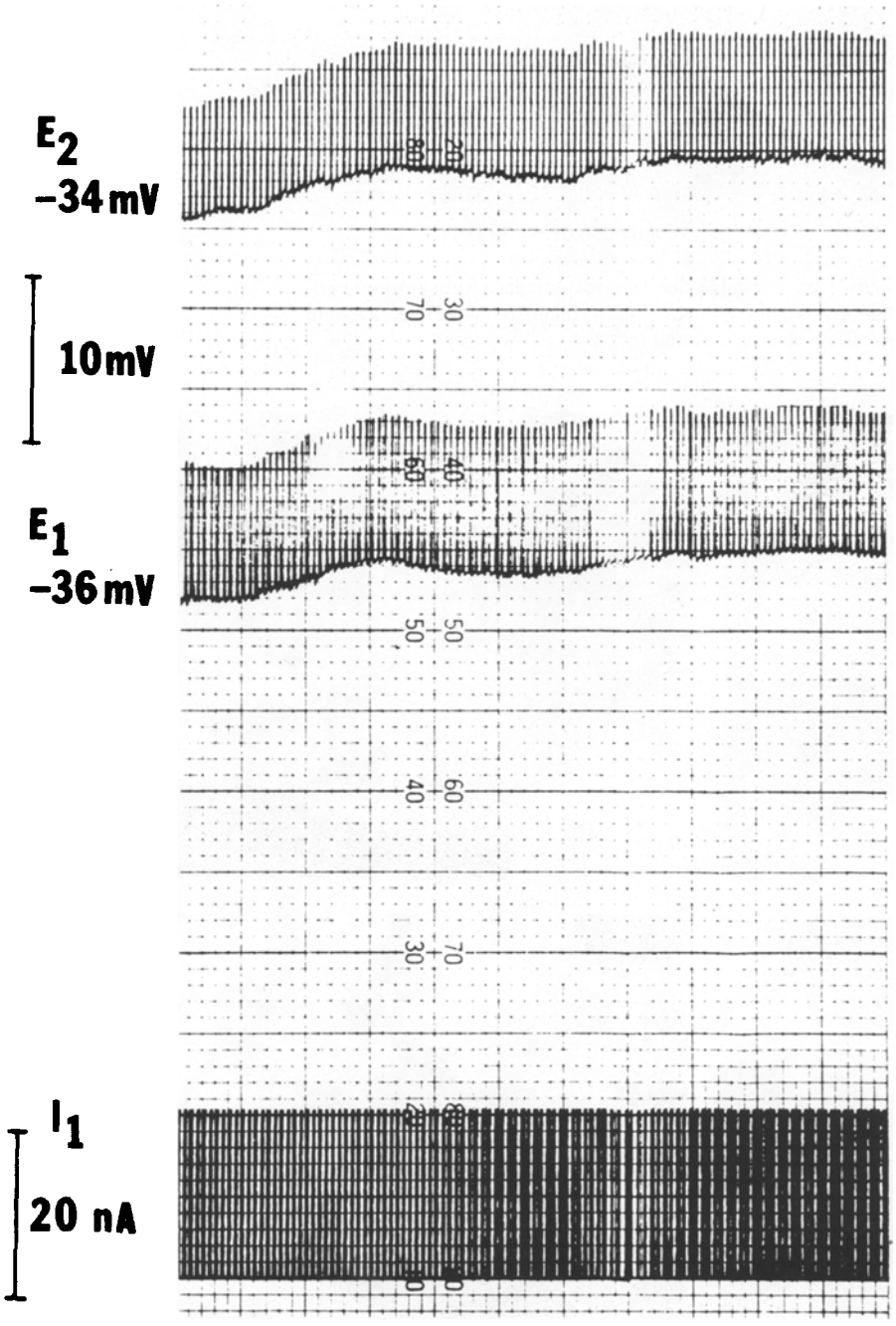

Fig. 7. Electrical coupling of the oocyte pair shown in Fig. 6. Coupling ratio $V_2/V_1 = 0.8$

we were able to freeze-fracture in the contact area showed gap junctions as well as tight junctions (Fig. 8).

Reversible Uncoupling of Channels

It has been shown that electrical coupling of cells via typical cell-cell channels can be reduced reversibly by adding carbon dioxide to the bath fluid (Turin & Warner, 1977; Rose & Rick, 1978; Spray, Harris & Bennett, 1981). In order to test whether the observed electrical coupling between oocytes was due to typical cell-cell channels we supplied 100% CO_2 to the bath. Figure 9 shows that, with a short delay, the membrane potential decreases by several millivolts and that the cells subsequently uncouple. After removal of the CO_2, electrical coupling gradually reappears. This reversible uncoupling is observed with both endogenous (control) and mRNA-induced cell-cell channels. It is note-

Fig. 8. Electron micrograph of the interface of a control oocyte pair exhibiting endogenous cell-cell coupling. Gap junctions (arrow) and extensive tight junctional strands can be observed. Magnification: ×44,800

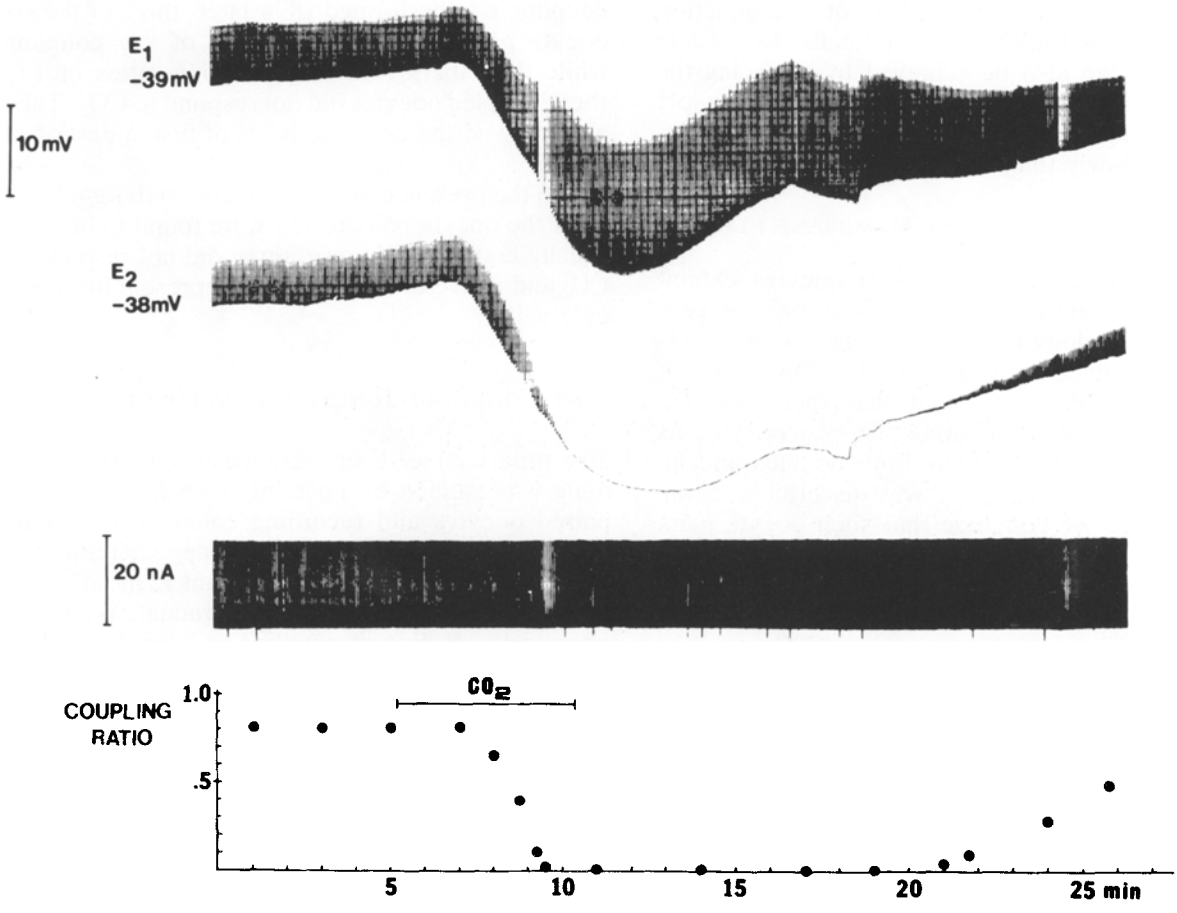

Fig. 9. Effect of CO_2 on the electrical coupling of an mRNA-injected oocyte pair. At the time indicated, the recording chamber was continuously perfused with Barth saline saturated with 100% CO_2. With a delay of a few minutes (partly attributable to fluid exchange) the electrotonic potential (V_2) declines and finally disappears while the input resistance of the current-injected cell increases (V_1). The coupling ratio drops from 0.8 to zero and reappears slowly upon removal of CO_2

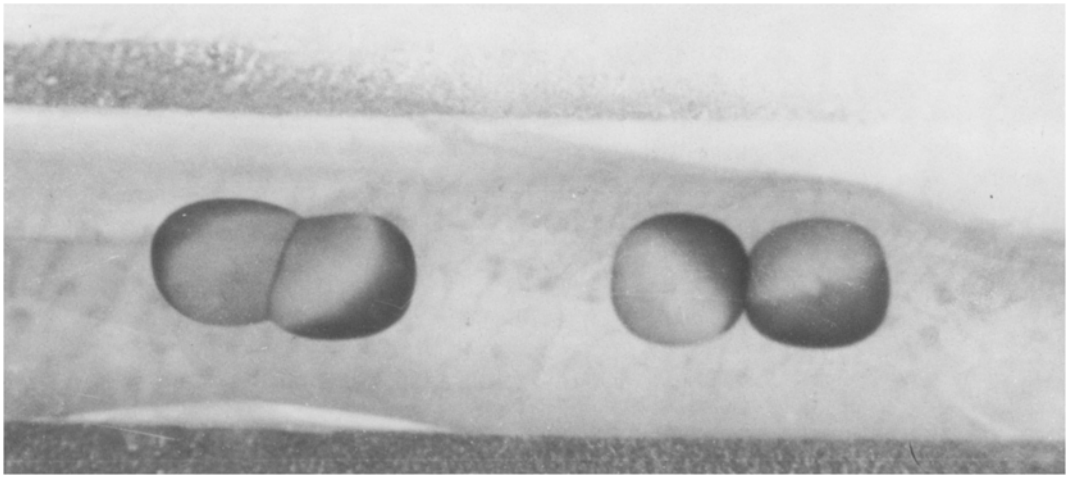

Fig. 10. Pairs of oocytes. (Right) typical appearance; (left) fused oocytes lacking sharp angles at original contact site

worthy that in oocyte triplets, where control cells showed coupling to their mRNA-injected neighbors, the uncoupling of this junction by CO_2 consistently preceded the uncoupling of the junction between the two mRNA-injected cells. Reversible uncoupling can also be achieved by replacing the bath with one containing 45 mM sodium acetate, pH 7.0 (replacing NaCl). Sodium acetate exerts its effect more slowly than CO_2.

Artifactual Coupling by Membrane Fusion

Under certain conditions, paired oocytes exhibit extreme coupling ratios of 1.0. Such pairs have a typical morphology as shown in Fig. 10; they cannot be uncoupled by CO_2 (Fig. 11). Dissection of such oocyte pairs indicated that there was no plasma membrane separating the two oocytes as yolk was seen leaking from both oocytes and no submembrane pigmentation was discernible. From this evidence we conclude that such oocyte pairs are fused. Consequently, we used CO_2 routinely to test against such an artifact. Conditions favoring cell-cell fusion are: (i) longtime incubation exceeding 72 hr; (ii) temperatures above 25°C for more than 24 hr; (iii) conditions that are expected to reduce the protein content of the oocyte plasma membrane such as (a) proteolysis (trypsinization), or (b) inhibitors of transcription and translation (actinomycin D, cycloheximide). Earlier experiments with Cl-1D cells had hinted already that cycloheximide could cause artifactual coupling by cell-cell fusion (Azarnia, Dahl & Loewenstein, 1981). The present observation strongly supports this earlier finding.

Involvement of Protein Synthesis

For the reasons given above, experiments that include the drugs actinomycin D and cycloheximide must be evaluated with caution. When cycloheximide (10 μg/ml) was added to the oocytes at the time of mRNA injection, and recording of electrical coupling was performed 18 hr later, three of the six oocyte pairs tested were devoid of any coupling while the others exhibited coupling ratios of 1.0; these coupled oocytes did not respond to CO_2. They also showed the characteristics of fusion described above.

In the presence of actinomycin D (10 μg/ml), all 12 of the oocyte pairs tested were found to be electrically coupled. Of these, three did not respond to CO_2 and, therefore, presumably represent fused oocyte pairs.

Time Course of Junction Development

The time course of appearance of electrical coupling was studied by injecting mRNA into freshly paired oocytes and recording coupling at various time intervals. Coupling did not appear until 4 hr later. It increased rapidly until about 12 hr, at which time further increase was more gradual. We never observed a decline of coupling at later times (Fig. 12). As has been shown in other studies, mRNA injected into *Xenopus* oocytes appears to be stable and functions in translation for very long periods of time (Gurdon, Lingrel & Marbaiz, 1973). Interpretations of measurements taken at very late time points (beyond 72 hr) are complicated: those pairs that survive show coupling ratios of 1.0, which are unaffected by CO_2 and exhibit the morphological characteristics of cell-cell fusion.

The time course of appearance of cell-cell junctions is determined by two major processes: (i) synthesis and intracellular transport of channel components, and (ii) assembly of functional cell-cell channels at the membrane level. In order to distin-

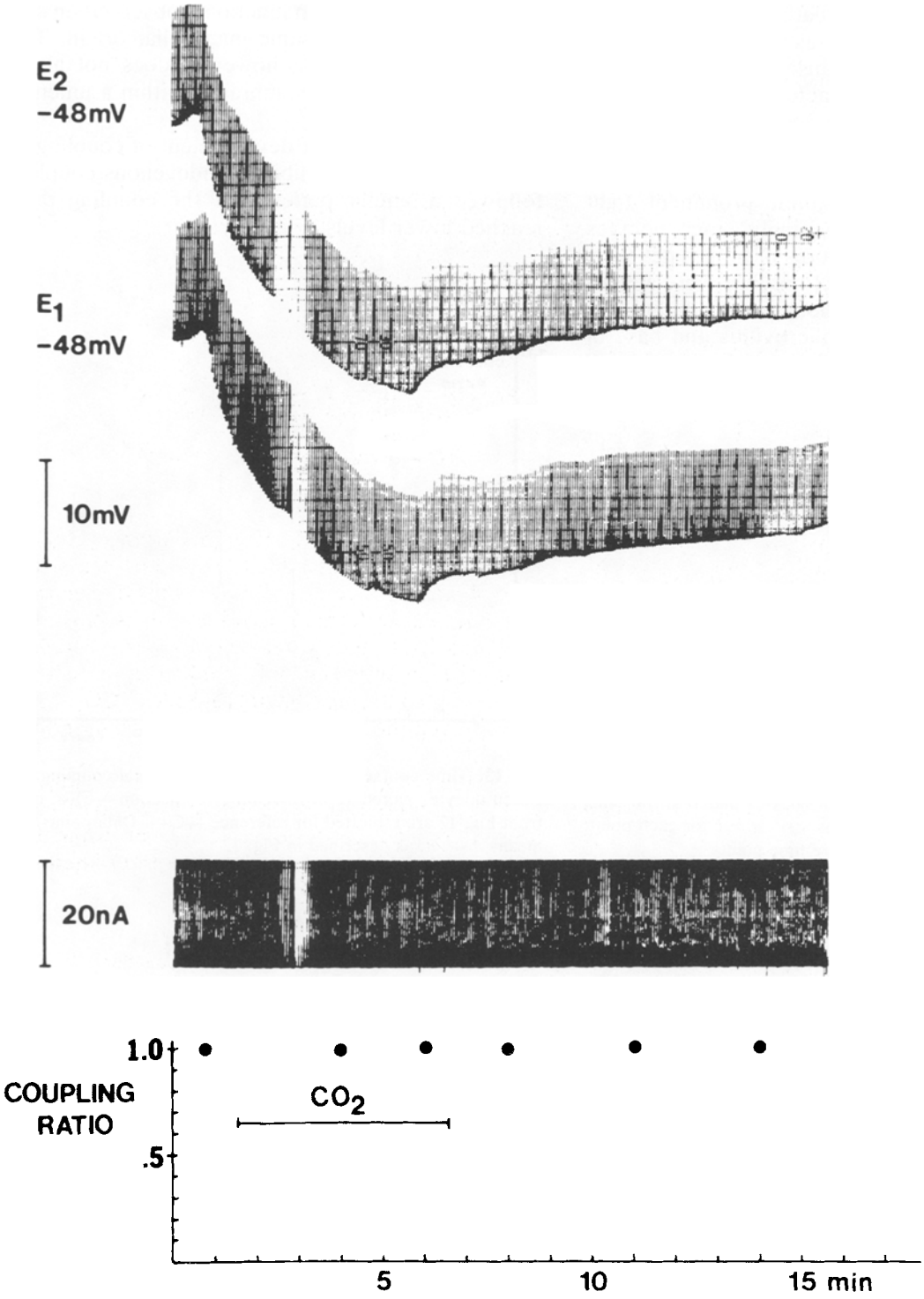

Fig. 11. Lack of uncoupling by CO_2 in an oocyte pair with altered morphology (*see* Fig. 10)

guish between these two processes, we injected single oocytes with myometrial mRNA and delayed the pairing by 12 hr. During that time period we expected the oocytes to synthesize and accumulate channel precursors in a readily available pool. To our surprise, however, delayed pairing had only little, if any, effect on the time course of junction formation (Figs. 13 and 14). Since oocytes are known to exhibit endocytotic activity, even in the isolated state, it is conceivable that a large pool of channel precursors cannot build up. To test this hypothesis, we added cytochalasin B, an inhibitor of

endocytosis, to the oocyte bath. No effect on the rate of junction formation was observed (data not shown). It was mainly because of this obervation that we performed freeze-fracture electron microscopy on single oocytes. We deliberately chose oocytes from a batch with a high background of endogenous cell-cell channels. In addition to gap junctions, such oocytes exhibit prominent tight junctions when paired. Surprisingly, single oocytes can exhibit junctional structures of both types (Fig. 15). In the case of tight junctions, we were able to trace the origin of the junctions to rebounding microvilli (junction between microvillus and base of oocyte cell membrane). It is conceivable that all junctions (including gap junctions) observed on single oocytes have the same microvillar origin. The freeze-fracture process, however, does not allow identification of both membranes within a junction in each case.

The time course of development of coupling in batches of oocytes exhibiting endogenous coupling followed a similar pattern but the coupling ratio reached lower levels (Fig. 12).

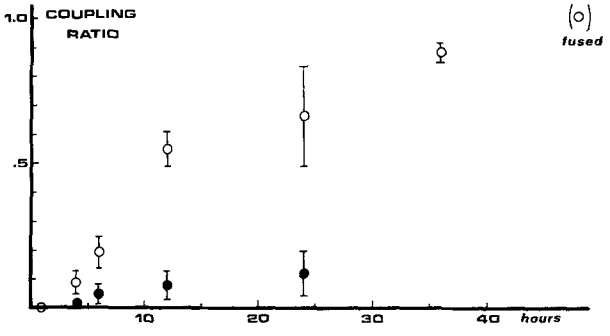

Fig. 12. Time course of development of electrical coupling between paired oocytes. Oocyte pairs injected with mRNA immediately after pairing —○—; control oocytes paired at the same time —●—. Data points are means ±SE. $n \geq 3$ for each point; each oocyte pair yields two or more time points

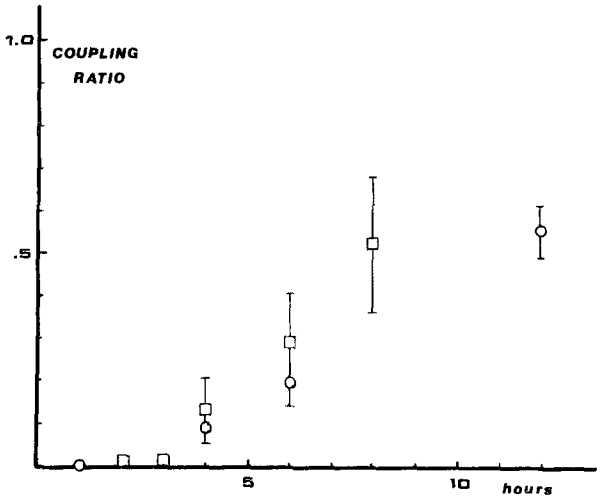

Fig. 13. Time course of development of electrical coupling between oocytes paired 12 hr after mRNA injection —□—; data from Fig. 12 are replotted for reference —○—. Data points are means ±SE. n, as described in Fig. 12

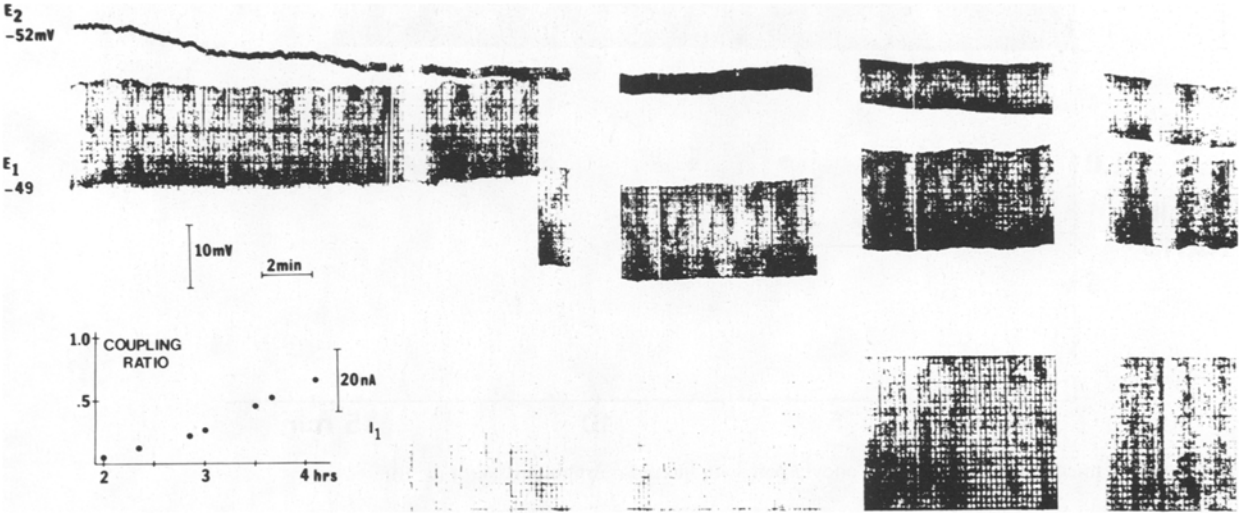

Fig. 14. Electrical coupling in a pair of oocytes. mRNA was injected into the oocytes 12 hr before they were paired. The electrical recording of the pair shown started 2 hr after pairing and extended over another 2-hr period (the intervals between the recordings are 30 min). Current pulses (I_1) injected into oocyte 1 hyperpolarizes this membrane (V_1) and the membrane of the neighboring oocyte (V_2). Over this recording period of 2 hr the coupling ratio, expressed as V_2/V_1, increases progressively, indicating recruitment of cell-cell channels

Fractionation of mRNA

As a first step in purifying the mRNA species responsible for the induction of cell-cell channels in oocytes, we sedimented mRNA from estrogen-stimulated myometrium through a denaturing sucrose gradient. The capability to induce cell-cell channels in *Xenopus* oocytes was found to be restricted to a few fractions of mRNA in the medium molecular weight range (Fig. 16).

Discussion

Evidence for the Induction of Typical Cell-Cell Channels

Microinjection into *Xenopus* oocytes of mRNA isolated from estrogen-stimulated rat myometrium results in the induction of cell-cell channels between paired oocytes. This conclusion is based on the observed increase in electrical coupling between paired oocytes. The coupling shows the characteristics of typical cell-cell channels: (i) size-limited intercellular diffusion of tracer molecules as indicated by cell-to-cell passage of ^{35}S-sulfate ions but not of ^{125}I-labeled transferrin; (ii) the appearance of clusters of membrane particles at membrane contact sites typical for gap junctions; and (iii) the reversible uncoupling by carbon dioxide. The induction of cell-cell coupling in oocytes can be observed against a zero background or as an easily discernible increase over intrinsic coupling, depending on the physiological state of the batch of oocytes.

These results confirm our previous conclusions obtained in an earlier study in which the same type of mRNA preparation induced cell-cell coupling in a coupling-deficient tumorigenic cell line (Dahl et al., 1980, 1981). In these studies, mRNA was introduced into the recipient cell via liposomes. With that method only a limited number of mRNA molecules could be introduced into the recipient cell. Although the incidence of electrical coupling (the number of cells receiving mRNA and expressing it)

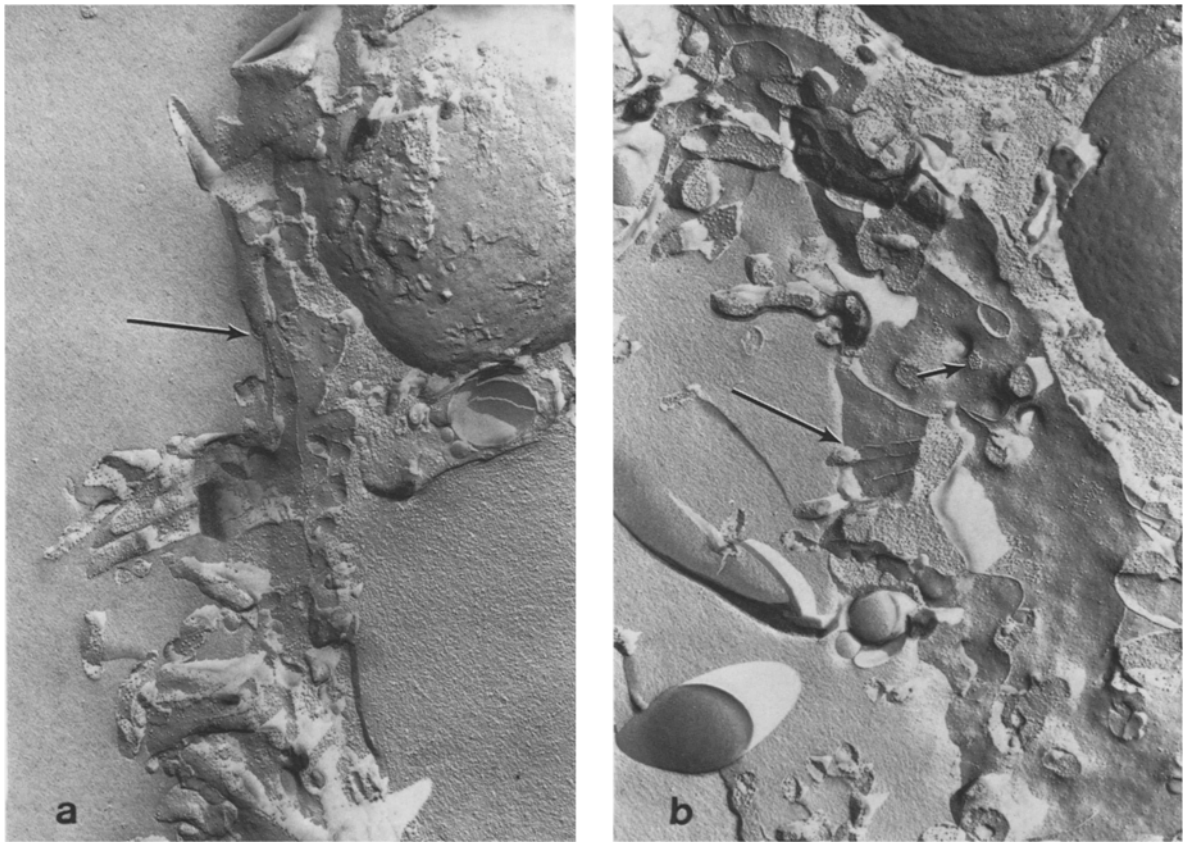

Fig. 15. Electron micrographs of junctional structures on single oocytes. (*a*) Tight junctions (large arrow) can be seen between a microvillus and the base of the oocyte membrane. (*b*) The oocyte surface exhibits tight junctions (large arrow) and gap junctions (short arrow). While the tight junction can be identified as an interaction between a microvillus and the oocyte membrane, a membrane partner for the gap junctional structure cannot be identified due to the course of the fracture plane. Magnification: ×28,000

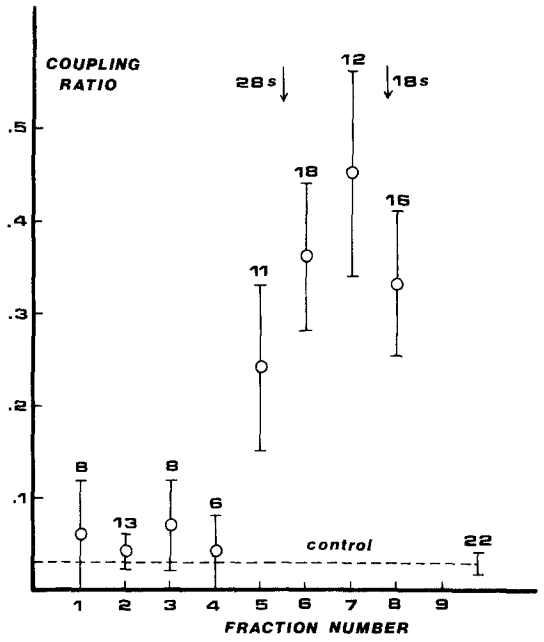

Fig. 16. Fractionation of mRNA on a denaturing sucrose gradient; 4.6 to 22% sucrose in 70% formamide. Sedimentation was at 35,000 rpm for 24 hr at 25°C in a Spinco SW-41 rotor. The mRNA in each of the nine fractions was precipitated with ethanol and dissolved in 20 μl of distilled water. The number above each point indicates the number of oocyte pairs tested. Data points are means ±SE. ^{3}H-labeled yeast ribosomal RNA served as molecular weight markers in a parallel gradient (sedimentation positions of 18*s* and 28*s* rRNA are indicated by arrows)

usually was high (60–70%, occasionally 100%), the number of the protein molecules expressed in the cell membrane was low as indicated by the observed coupling ratios (often less than 0.1). This inefficiency of mRNA delivery combined with the high cost of mRNA purification from rat uterus makes our new approach, the microinjection of mRNA into *Xenopus* oocytes, a superior system for assaying channel-specific mRNA on a large scale. An additional advantage of the oocyte system is its simplicity. It is a two-cell system with symmetrical geometry at the junction. This makes it easy to measure junctional conductance with a high degree of confidence. Furthermore, the junction's functional state can be manipulated easily. For example, modulators of channel conductance can be injected into the oocyte near the junctional site and their effects studied. The large number of channels induced in paired oocytes may also allow biochemical analysis of the channel protein. Such analysis has been successfully performed on other proteins synthesized in oocytes in response to the injection of foreign mRNA (reviewed by Lane and Knowland, 1975). These advantages outweigh the time-consuming procedure of oocyte stripping.

The fact that oocytes, in contrast to Cl-1D cells, can show intrinsic coupling is not necessarily a disadvantage, as will be discussed later. It only appears in certain batches and can be distinguished from the coupling that is induced by injected mRNA quantitatively and qualitatively. From the published literature one would not have expected mature oocytes (stage VI) to express cell-cell channels when paired. For mammalian oocytes it has been reported that the junctions with the follicle cells disappear with maturation (Gilula, Epstein & Beers, 1978). For *Xenopus* oocytes it has been stated that HCG treatment is required for the formation of oocyte-follicle cell junctions (Browne, Wiley & Dumont, 1979). The existence of intrinsic cell-cell channels in oocytes, therefore, is surprising. There are at least three possible interpretations for this observation. First, the intrinsic junctions may be translated from leftover mRNA used for the synthesis of oocyte-follicle cell junctions. Second, the oocyte might contain mRNA for the synthesis of cell-cell channels to be used after fertilization and in early embryonic development during which time little transcriptional activity is observed. Third, oocytes are known to contain a large variety of different mRNA species, some of which may be translated into proteins in a more or less random fashion. For example, it has been shown that oocytes randomly exhibit membrane receptors with no apparent function at that stage or during early embryonic development (Kusano, Miledi & Stinnakre, 1982).

We consider this third interpretation unlikely because we observed that oocytes obtained from a single frog behaved similarly. There was no evidence for random appearance of intrinsic cell-cell coupling. Either most of the oocytes exhibited coupling or none of them did. With regard to the first two interpretations, we cannot discriminate between the two mechanisms at this time. A decisive answer would require extensive morphometric analysis of oocyte-follicle cell junctions and oocyte-ooctye coupling measurements combined with precise staging of oocyte maturation.

The Time Course of Cell-Cell Channel Formation

The time course of appearance of electrical coupling between paired oocytes after mRNA injection closely follows the pattern originally observed for the induction of junctions in Cl-1D cells (Dahl et al., 1980, 1981). Within the time span where measurements could be taken (later, fusion occurs), the oocytes showed a continuous increase in cell-cell coupling. This is in contrast to Cl-1D cells where mRNA infusion resulted in a transient appearance

of cell-cell coupling lasting not much longer than 24 hr. We concluded at that time that the infused mRNA was short-lived and that the electrical coupling reflects the functional lifetime of the cell-cell channel. Subsequently another laboratory, using a completely different approach, reported essentially the same turnover time for the 27,000-dalton membrane protein considered to represent the major channel protein (Fallon & Goodenough, 1981). The oocyte system, as used in the present study, cannot give data on the lifetime of cell-cell channels. This could be explained solely by the known longevity of the injected mRNA in the oocyte (Gurdon et al., 1973) and does not necessarily imply a different half-lifetime of the channels proper.

The appearance of electrical coupling between paired oocytes can be regarded as the result of a series of events (Loewenstein, 1981; Chao, Young & Poo, 1981): (i) synthesis of channel proteins; (ii) intracellular shuttling; (iii) intramembrane diffusion of either subunits or assembled hemichannels; (iv) interlocking of bimembranous components (subunits or hemichannels); and (v) the opening of the cell-cell channel forming an aqueous pathway between two cells. We have tried to dissect parts of this complex pathway by delaying the pairing of oocytes after mRNA injection. This was done with the expectation that channel components might accumulate in the oocyte membrane and be available for the final steps of channel formation. Surprisingly, delayed pairing did not accelerate channel formation significantly. Several possible explanations come to mind: (i) Insertion of channel precursors into the plasma membrane may require the signal of membrane-membrane contact. (ii) The lifetime of precursors could be shorter than that of completed channels (the formation of a complete cell-cell junction might stabilize the channel proteins in the membrane). (iii) Unspecific endocytosis of membrane proteins might deplete channel precursors. (iv) The steps leading to channel formation following the insertion of channel precursors into the plasma membrane could be rate limiting.

We have no evidence for or against possibilities (i) and (ii). Explanation (iii) is unlikely in light of the observation that cytochalasin B has no effect on the rate of junction formation. Explanation (iv) is supported by the observation of recruitment of intrinsic channel precursors by mRNA-injected oocytes in the triplet configuration. Observations obtained on Cl-1D cells, on the other hand, indicated that synthesis and passage up to the Golgi apparatus require almost 50% of the total time required for functional channels to appear (G. Dahl, *unpublished results*). In the oocyte this process should be even slower due to the lower temperature, greater transport distances, etc. Thus the steps from membrane insertion to formation of a functional channel should only be rate limiting when the number of precursors in the membrane is low and consequently the probability of bimembranous interaction is affected.

Although these mechanisms may explain the failure of delayed oocyte-pairing to accelerate junction formation appreciably, there is another possible mechanism that might limit the number of channel precursors in the membrane. We observed that a single oocyte is capable of forming junctions with itself. For example, a rebounding microvillus can form a junction with the base of the oocyte membrane. Such a mechanism would prevent, or reduce the extent of, accumulation of free channel precursors in the plasma membrane. We have been able to identify oocyte-oocyte membrane junctions within single oocytes only with regard to tight junctions. For this study we deliberately chose batches of oocytes exhibiting intrinsic coupling which we knew was paralleled by the appearance of tight junctions and gap junctions. The nature of the freeze-fracture process is less favorable for identifying membrane partners of gap junctions. We have observed particle clusters typical for gap junctions in these single oocytes, too. Unfortunately, in none of them did the fracture plane change within the junction (an unlikely event) so as to allow us to identify the presence and nature of the contacting membranes. In light of the finding that tight junctions can be formed on rebounding microvilli, it is more reasonable to expect such a mechanism to apply for gap junctions as well instead of postulating the existence of half-junctions.

Size of mRNA Responsible for Cell-Cell Channel Induction

The expression of cell-cell channels in the oocyte membrane obviously is the result of the injection of myometrial mRNA. However, what is the nature of this mRNA? There are two possibilities: either the mRNA codes directly for the channel proteins or it codes for a regulatory protein which in turn causes the formation of functional cell-cell channels in the oocyte membrane. If the latter is true, one would have to argue that such a regulatory protein is also responsible for the upregulation of cell-cell channels in myometrium.

The size of the mRNA fraction capable of inducing cell-cell channels is surprisingly large considering that a 27,000-dalton protein is thought to be the major channel protein. If it codes directly for the channel protein, this apparent contradiction could be explained by large untranslated regions in the mRNA, extensive posttranslational processing, or a combination of both. On the other hand, the

major channel protein may be considerably larger. In fact, it has been reported recently that heart gap junctions consist of 47,000-dalton subunits (Manjunath, Goings & Page, 1984). Furthermore, antibodies raised against the 27,000-dalton protein from liver gap junctions were found to react with a 47,000-dalton protein (Henderson & Weber, 1982). This uncertainty about the nature of the major cell-cell channel protein reemphasizes the importance of a functional assay which the oocyte system presented here seems to represent.

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