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Abnormal development and dye coupling produced by antisense RNA to gap junction protein in mouse preimplantation embryos

(compaction/intercellular communication/blastocoel formation/excluded blastomere)

ARTURO BEVILACQUA*†, RITA LOCH-CARUSO‡, AND ROBERT P. ERICKSON*§¶

*Department of Human Genetics, and §Pediatrics and Communicable Diseases, School of Medicine and ‡Department of Environmental and Industrial Health, School of Public Health, University of Michigan, Ann Arbor, MI 48109

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ABSTRACT Antisense RNA to the 27/32-kDa rat liver gap junction (GJ) protein was used to explore the role of GJs in preimplantation embryos. When all blastomeres of two- and four-cell embryos were injected with GJ antisense RNA, the percentage of embryos compacted at 60 hr of development was reduced to less than 20%, while 90% of uninjected embryos and 75% of embryos injected with an unrelated RNA were compacted. When most cells of compacted eight-cell embryos were injected with the GJ antisense RNA, 20% of the embryos were decompacted and only 5% had developed to the blastocyst stage at 90 hr, when blastulation had occurred in 90% of the control embryos. When antisense RNA was injected in one blastomere of four-cell embryos, 40% of the embryos presented a large cell that was not included in the compacted embryo at the time of compaction, and an additional 30% of the embryos had two smaller, excluded blastomeres. These excluded cells were identified as the injected cell with a rhodamine-conjugated dextran marker. To assess effects on junctional communication, one blastomere of some embryos was injected with Lucifer yellow, a GJ-penetrating dye, at various times after a blastomere was injected with antisense RNA. The dye was visible in the whole cell mass of control embryos, but it was excluded from a portion of experimental embryos when the delay between the RNA and the Lucifer yellow injections was 1 hr or longer.

The dramatic changes in organization and complexity that occur during preimplantation development of the mouse embryo require the establishment of a mode of intercellular communication (1). Gap junctions (GJs) may play an important role in this coordinated process by providing a pathway for the exchange of small molecules and ions. Gap junctional communication in mouse embryos is first detected during the late eight-cell stage, at the time of compaction (2–9). Injection of an antibody to GJ protein at this time results in developmental abnormalities including blastomere exclusion and delays in blastulation (10).

As previously reported, antisense RNA or DNA can inhibit specific gene expression in mouse oocytes (11) and preimplantation embryos (12, 13). We now report using this technique to study the role of GJ synthesis in early embryogenesis of the mouse. We analyzed the effects of antisense RNA on two morphogenetic processes easily identifiable in early embryos: compaction, the cell flattening that occurs at the eight-cell stage; and blastulation, the formation of the blastocoel cavity, which takes place two cell cycles later. Effects of the antisense RNA on junctional communication were confirmed with the GJ-penetrating dye, Lucifer yellow (LY). Our results confirm the developmental anomalies obtained in a previous study with an antibody to GJ protein and suggest further that intercellular communication in postcompaction embryos is actively maintained through the rapid transcription and translation of GJ mRNAs.

MATERIALS AND METHODS

Chemicals. Pregnant mare serum gonadotropin, human chorionic gonadotropin, and chemicals were from Sigma. A cDNA for the 27/32-kDa rat liver gap junction protein (14), which contains the complete coding region and both 5′ and 3′ untranslated sequences of the gene, was the gift of David Paul (Harvard University, Cambridge, MA). pGEM vectors and *in vitro* transcription kits were from Promega; restriction enzymes and other enzymes used for subcloning were from Bethesda Research Laboratories or from Boehringer Mannheim; and [^{32}P]CTP was from Amersham. LY and rhodamine-conjugated polyanionic dextrans (M_r 10,000) were from Molecular Probes.

Animals. Animals were from The Jackson Laboratory or were bred from their stock in our colony. Twenty-one-day-old (C57BL/6J × SJL/J) F_1 female mice were superovulated by intraperitoneal injections of 5 units of pregnant mare serum gonadotropin followed by 5 units of human chorionic gonadotropin 48 hr later and were then mated with F_1 males of the same cross. The time of coitus and fertilization was assumed to be at midnight after mating.

Embryo Culture. Whitten's medium (15) was supplemented with 0.1 mM EDTA (medium M1) or modified by substitution of bicarbonate with 20 mM Hepes for embryo manipulation (medium M2). Embryos were collected 12 hr postcoitum in medium M2 containing hyaluronidase at 0.5 mg/ml, washed in M2, and cultured in M1 or M16 at 37°C in an atmosphere of 5% O_2/5% CO_2/90% N_2 or 5% CO_2 in air.

RNA Preparation. The 1.5-kilobase (kb) cDNA for rat liver 27/32-kDa GJ protein was obtained as an *Eco*RI insert in pGEM-3, in both orientations. The DNA constructs were linearized with *Hin*dIII and used with phage T7 RNA polymerase for both sense and antisense RNA synthesis by the procedure of Melton *et al.* (16). β-Glucuronidase antisense RNA was similarly synthesized on a 0.35-kb subclone of the cosmid cosB1. The RNAs were purified and resuspended in TE (10 mM Tris·HCl/1 mM EDTA, pH 7.5) under conditions as described (13) at a concentration of 1 μg/μl.

Embryo Injections with RNA. Embryos were collected and transferred to a 50-μl drop of M2 medium under mineral oil and manipulated as described by Hogan *et al.* (17). A total of approximately 20 pg of the various RNAs were injected in each embryo. Two-cell embryos were collected 36 hr postcoitum, and both blastomeres were injected with approximately 10 pg of antisense RNA; the embryos were then cultured until the time of normal compaction (60–65 hr

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Abbreviations: GJ, gap function; LY, Lucifer yellow.
†Present address: Department of Psychology, University "La Sapienza" of Rome, 00185 Rome, Italy.
¶To whom reprint requests should be addressed.

Table 1. Effects on compaction of injecting GJ antisense RNA into each cell at the two- and four-cell stages

RNA injected	Stage	*n*	Embryos, no. (%)		
			Compacted	Uncompacted	Degenerated
GJ antisense	Two-cell	32	6 (19)	15 (47)	11 (34)
β-Glucuronidase antisense	Two-cell	18	15 (83)	0	3 (17)
None	Two-cell	25	23 (92)	0	2 (8)
GJ antisense	Four-cell	21	3 (14)	12 (57)	6 (29)
β-Glucuronidase antisense	Four-cell	8	6 (75)	0	2 (25)
None	Four-cell	21	20 (95)	1 (5)	0

postcoitum) and scored for abnormalities. Four-cell embryos were collected 42 hr postcoitum, and each blastomere was injected with approximately 5 pg of RNA; the embryos were cultured until the eight-cell stage and scored. In some cases, 20 pg of RNA was injected into eight-cell embryos 65 hr postcoitum, with the solution administered equally to at least six blastomeres in each embryo; the embryos were cultured until the time of normal blastulation (90 hr postcoitum) and scored. In other cases, 5 pg of RNA was injected into one blastomere of four-cell embryos collected 42 hr postcoitum; the embryos were cultured until the eight-cell stage and scored.

Combined Antisense RNA and Rhodamine-Dextran Injections. Antisense RNA to GJ protein was prepared as described above, and the RNA solution (1 μg/μl) was diluted 50:50 with a 10% (wt/vol) aqueous solution of rhodamine-dextran. Approximately 10 pl of this solution was injected into a single blastomere of four-cell embryos. The embryos were examined 18 and 36 hr after injection by using a Nikon Diaphot epifluorescence microscope equipped with filters for rhodamine fluorescence (a 510- to 569-nm excitation filter and a 590-nm barrier filter).

Dye Transfer Studies. RNA (1–3 pg) was injected into a single blastomere of compacted 8-cell or 16-cell embryos. The embryos were cultured at 37°C. After time periods of 10 min to 3 hr, approximately 5 pl of 5% (wt/vol) LY in TE was injected into a single blastomere of each embryo. The embryos were kept at 37°C for 5 to 10 min and then observed with a Zeiss III RS epifluorescence microscope equipped with an FITC filter set. Subsequent observations were performed 45–60 min after LY injection, with the embryos maintained at 37°C between the two observations.

RESULTS

Toxicity of GJ Antisense RNA. We used β-glucuronidase antisense RNA as a control in most of our experiments because it is the RNA with the largest effect on the embryo that we had previously found; GJ sense RNA was used as a control in other experiments. The GJ antisense RNA resulted in a higher percentage of degenerated embryos than did the control injections in these experiments. The combined data in Tables 1–3 show that 36 of 136 embryos injected with GJ antisense RNA degenerated, 5 of 86 embryos injected with antisense RNA to β-glucuronidase degenerated, and 18 of 155 noninjected embryos degenerated. These differences are highly significant ($\chi^2 = 9.62, P < 0.01$ against noninjected; $\chi^2 = 13.58, P < 0.01$ against injected with antisense RNA to β-glucuronidase). It is possible that this toxic effect is due to the specificity of the antisense RNA in inhibiting expression of essential gap junctions.

Effects of GJ Antisense RNA on Compaction. Embryos were injected at the two- or four-cell stage (Table 1). After injection, the embryos were cultured until the normal time of compaction (65–70 hr postcoitum) and then scored. All uninjected, viable two-cell embryos underwent compaction during this time, and all the viable two-cell embryos injected with β-glucuronidase antisense RNA as a control underwent compaction. In contrast, only 28% of the viable two-cell embryos injected with GJ antisense RNA underwent compaction. When GJ antisense RNA was injected in four-cell embryos, a similar low degree of compaction (20%) was observed.

Effects of GJ Antisense RNA on Blastulation. Eight-cell embryos were injected 60–65 hr postcoitum and then cultured (Table 2). At 90 hr postcoitum, 89% of the uninjected control embryos and all of the control embryos injected with β-glucuronidase antisense RNA were at the blastocyst stage. In contrast, only 5% of the viable embryos injected with GJ antisense RNA had developed a blastocoel. Furthermore, 19% of the injected embryos were still uncompacted; these embryos remained arrested with further culture. After an additional 24 hr of culture, all of the embryos that were compacted at 90 hr postcoitum had blastulated.

Effects of GJ Antisense RNA on Single Blastomeres. Injection of single blastomere in four-cell embryos was performed 40–42 hr postcoitum with scoring of the embryos at the normal time of compaction (Table 3). This manipulation produced embryos in which either one large cell (39%) or two

Table 2. Effects on blastulation of injecting GJ antisense RNA into most of the blastomeres of eight-cell embryos

RNA injected	Stage	*n*	Embryos, no. (%)			
			Blastocysts	Compacted	Uncompacted	Degenerated
GJ antisense	Eight-cell	21	1 (5)	8 (38)	4 (19)	8 (38)
β-Glucuronidase antisense	Eight-cell	29	29 (100)	0	0	0
None	Eight-cell	18	16 (89)	2 (11)	0	0

Table 3. Effects of injecting GJ antisense RNA into one blastomere of four-cell embryos

RNA injected	*n*	Embryos, no. (%)			
		Normally compacted	Compacted with excluded blastomere(s)	Uncompacted	Degenerated
GJ antisense	62	0	41 (66)	10 (16)	11 (18)
β-Glucuronidase antisense	31	24 (77)	0	7 (23)	0
None	91	70 (77)	0	5 (5)	16 (18)

Table 4. Results of coinjection of rhodamine-dextran and GJ antisense RNA

			Embryos, no.				
Injection	Rhodamine fluorescence observed	*n*	Normally compacted	Compacted with intact excluded cells	Compacted with dead excluded cells	Uncompacted	Degenerated
Rhodamine-dextran	+	10	10	0	0	0	0
	−	10	10	0	0	0	0
GJ antisense	+	20	0	11*	0	8	1
RNA + rhodamine	−	20	6	1	7	4	2

*Rhodamine fluorescence was limited to excluded cells.

smaller ones (27%) were excluded from the rest of the compacted embryo. As we never observed excluded blastomeres in control embryos, regardless of whether they were uninjected or were injected with control RNA, we propose that these excluded cells were those containing the GJ antisense RNA. The single large excluded cell may have been the undivided blastomere injected with the RNA. When two cells were excluded, these may have been the daughter cells of the injected cell or the injected cell and its sister blastomere, if cytoplasmic bridges were intact at the time of injection. Apparently, the noninjected blastomeres formed normal six-cell embryos with a frequency of compaction (66%) similar to controls (77%).

Coinjection of GJ Antisense RNA and Rhodamine-Dextran into a Single Blastomere. To demonstrate that the excluded blastomere(s) were those injected with GJ antisense RNA, we injected a single blastomere of four-cell embryos with a mixture of the GJ antisense RNA and rhodamine-dextran, a marker previously used with great success in the mouse preimplantation embryo (18). The high molecular mass of the rhodamine-dextran would prevent it from passing through gap junctions, although it would readily diffuse to sister blastomeres through cytosolic bridges remaining from cytokinesis (1). From a total of 20 successfully injected embryos, 11 embryos compacted (Table 4). Each of these compacted embryos exhibited fluorescent cells that were excluded from the remainder of the compacted embryo, while fluorescence was not observed in any cells within the compacted embryos (Fig. 1). In the majority of cases (8 of 11 compacted embryos), two fluorescent cells were excluded. In one compacted embryo, only one fluorescent cell was excluded. In two additional compacted embryos, three or four cells were excluded: all excluded cells were fluorescent in one of these embryos, but only two of the excluded cells were fluorescent in the other embryo. Eight embryos successfully injected with the RNA/rhodamine solution failed to compact, with less than six cells per embryo, suggesting that the injection arrested embryonic development. One injected embryo was degenerated with two fluorescent uncompacted cells. Twenty other embryos injected with the RNA/rhodamine solution failed to show fluorescence. We suggest that either these embryos were not penetrated by the injecting pipette, the dye faded, or the cells were injured by the injection and leaked the dye. Of 20 control embryos injected with rhodamine-dextran, only 10 embryos displayed fluorescence, yet all 20 compacted normally. These data show that the cells excluded from compacted embryos were those cells containing the mixture of GJ antisense RNA and rhodamine-dextran.

Effects of GJ Antisense RNA on GJ Communication. The fluorescent dye LY was used to study the effect of antisense RNA on GJ communication of compacted embryos (Fig. 2 and Table 5). LY was injected into a single blastomere at various times (10 min to 3 hr) after injection of the RNA. At any time point, LY diffused rapidly (<10 min) to all of the other blastomeres in all but three of the 38 non-RNA-injected embryos. The dye also spread rapidly to all blastomeres of the embryos injected with LY 10 min after the RNA, both in the case of GJ antisense ($n = 5$) and control ($n = 4$) RNA. However, when LY was injected at least 30 min after GJ antisense RNA, dye transfer was restricted in the majority of embryos: at 30 min and 1 hr after RNA injection, only 36% and 24% of embryos, respectively, showed fluorescence in all blastomeres. At later times, 2 and 3 hr, no embryos had fluorescence in all blastomeres ($n = 7$ and $n = 9$, respectively). In the majority of embryos injected with control (GJ sense or β-glucuronidase antisense) RNA, the dye transferred to all blastomeres: 69% of embryos at 30 min and 75% of embryos at 1 hr. Considering data from the GJ antisense RNA treatments, the incomplete dye transfer was generally characterized by restriction of dye to one or two cells (16 of 39) or exclusion of dye from one or two cells (18 of 39). In four embryos, dye was restricted to about one-half of the embryo, and it was limited to three cells in one embryo.

DISCUSSION

The biological effect of GJ antisense RNA injection is comparable to that reported previously with an affinity-purified

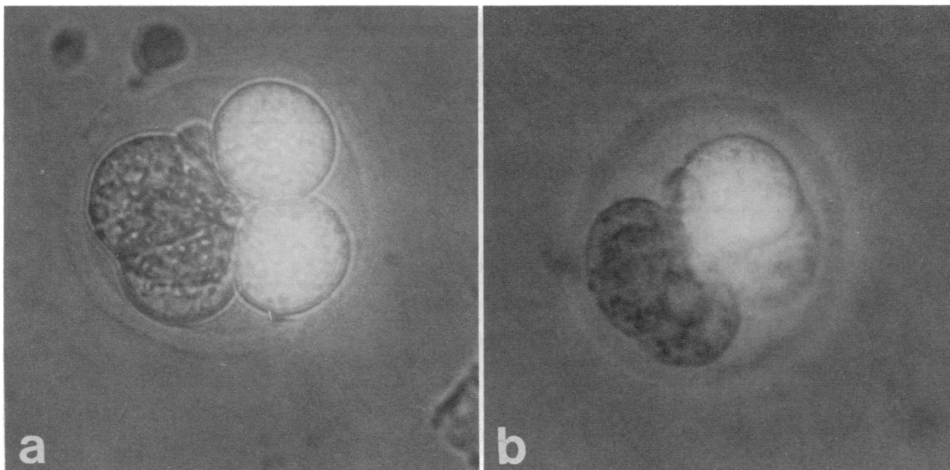

FIG. 1. Distribution of rhodamine-conjugated dextran in embryos that had one blastomere injected with a combination of rhodamine-dextran and GJ antisense RNA at the early four-cell stage. (*a*) Precompaction embryo 18 hr after injection, showing restriction of dye to two blastomeres. (*b*) Compacted embryo 36 hr after injection, showing two fluorescent excluded blastomeres.

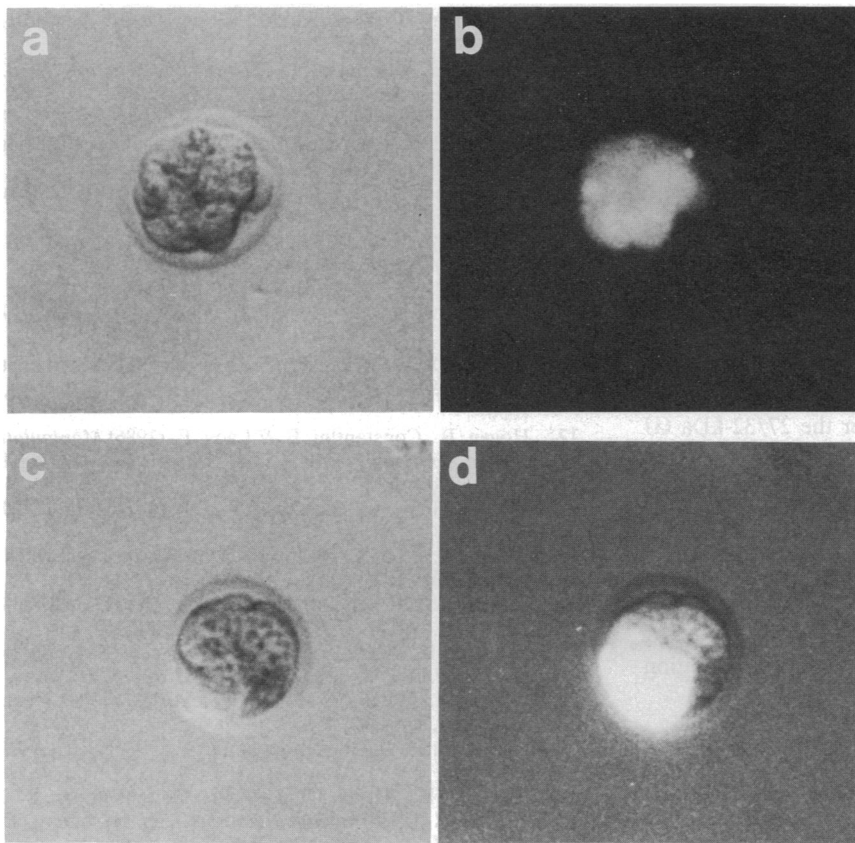

FIG. 2. Analysis of GJ communication by LY transfer. (*a*) Phase-contrast view of control embryo. (*b*) Fluorescence of LY throughout embryo 45 min after injection into a single blastomere of the control embryo. (*c*) Phase-contrast view of experimental embryo. (*d*) Fluorescence of LY injected 90 min after injection of antisense RNA into one blastomere (presumably the one injected with antisense RNA); the photograph was taken 60 min after injection of LY.

antibody (10): injection of antisense RNA or antibody resulted in exclusion of cells from the compacted embryo. The antibody presumably disrupts GJ function, whereas the antisense RNA presumably disrupts GJ synthesis. The effects on compaction of GJ antisense RNA seen in Tables 1 and 3 support the idea that functional gap junctions may be necessary for the maintenance of this state (10, 19). Interestingly, when the embryos injected with GJ antisense RNA were cultured for 1 or 2 days after the scoring, none of those that had failed to compact at the time of normal compaction developed further. These observations, and the overall toxicity of the GJ antisense RNA, suggest that GJ antisense RNA disrupts the normal pathway of junctional communication, which in turn produces the state of decompaction we have observed. The final result is a block in development. This kind of abnormality is strikingly similar to the lethal developmental condition described by Buehr *et al.* (19) in the embryos of the DDK strain of mice fertilized by foreign sperm: reduced junctional communication accompanied by spontaneous decompaction and embryonic death. Therefore, it appears that functional GJs are necessary at this time of development for the normal progression of embryogenesis in the mouse.

We attribute the observed temporary effect on blastulation to the injection of an insufficient amount of GJ antisense RNA. It was apparent that if embryos were able to compact after the injection, they would eventually form a blastocyst. More experiments will be needed to correlate junctional communication with the process of blastulation.

The experiment in which rhodamine-dextran was used to mark RNA-injected cells provided insight into the origin of the excluded cells. Because fluorescence was observed only in excluded cells, the results show that the excluded cells were those that received the GJ antisense RNA. However, in most cases more than one fluorescent cell was excluded, even though only one cell was injected with the solution. This observation may be explained by one of the two occurrences: either cytoplasmic bridges were present between sister blastomeres at the time of injection, allowing the RNA/rhodamine solution to freely pass to another cell (1), or the injected cell divided and redistributed the RNA/rhodamine solution to its daughter cells. The single case of an embryo with only one excluded fluorescent cell may be explained if either this embryo was more developed than the other embryos at the time of injection and the cytoplasmic bridges had resolved, or the injected cell failed to divide. In the case of the embryo with three or four excluded fluorescent cells, we suggest that at least one of the injected cells underwent cell division.

The dye-coupling results of Table 5 further suggest that GJs in 8- and 16-cell embryos are replaced every 30–60 min. This

Table 5. Effects of LY transfer of injecting GJ antisense RNA into one cell of compacted embryos

	No. of embryos with LY diffused through all blastomeres/ no. successfully injected with LY (%)				
RNA injected	10 min*	30 min*	1 hr*	2 hr*	3 hr*
GJ antisense	5/5 (100)	4/11 (36)	5/21 (24)	0/7 (0)	0/9 (0)
Control RNA†	4/4 (100)	9/13 (69)	6/8 (75)	ND‡	ND‡
None	1/1 (100)	12/13 (92)	8/10 (80)	7/7 (100)	7/7 (100)

*Time to LY injection.
†GJ sense RNA or β-glucuronidase antisense RNA.
‡ND, not determined.

turnover rate is more rapid than previously estimated for liver GJ proteins. Initial estimates of the half-life of the rat liver 27/32-kDa GJ protein were high, about 19 hr (20), although this value was later modified to 5–10 hr (21). Subsequent immunoblot experiments confirmed a shorter half-life of about 4 hr (22). Similar results were reported for the 26/27-kDa mouse liver GJ protein, with an estimated half-life of 5 hr *in vivo* (23) and 2–3 hr in cultured liver cells (24). The more rapid turnover rate we observed might be a developmental strategy to rapidly modulate junctional communication in cells that will soon start differentiation: in fact, during early postimplantation stages, the embryos progressively compartmentalize cell-to-cell communication concomitant with the differentiation of their cells in giant trophoblast, embryonic endoderm, and embryonic ectoderm (25, 26).

Although we have used the cDNA for the 27/32-kDa GJ protein to generate antisense RNA, it is possible that we are inhibiting the expression of other GJ mRNAs. Gerald Kidder (personal communication) has found evidence for "heart" connexin 43 mRNA but not for "liver" connexin 32 mRNA in postcompaction embryos. The 5′ coding regions of several GJ mRNAs show marked sequence homology (14, 27–31). Given this potential for cross-hybridization, the importance of the 5′ region in antisense inhibition (32), and the high molar excess of antisense RNA that we are able to inject, inhibition of expression of several forms of GJ RNA is likely.

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