17. S. R. Coughlin, W. M. F. Lee, P. W. Williams, T. M. Giels, L. T. Williams, *Cell* **43**, 243 (1985).
18. A. R. Frackelton, Jr., P. Tremble, L. T. Williams, *J. Biol. Chem.* **259**, 7909 (1984).
19. Y. Yarden *et al.*, *Nature* **323**, 226 (1986).
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Cloning and Expression of a *Xenopus* Embryonic Gap Junction Protein

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Gap junctions in the early amphibian embryo may play a fundamental role in the regulation of differentiation by mediating the cell-to-cell transfer of chemical signals. A complementary DNA encoding a gap junction present in *Xenopus* oocytes and early embryos has now been cloned and sequenced. This protein sequence is homologous to the well-characterized gap junction structural proteins rat connexin32 and connexin43. RNA blot analysis of total *Xenopus* oocyte RNA showed hybridization to a single 1.6-kilobase band. This messenger RNA is abundant in oocytes, decreases to levels below the sensitivity of our assay by stage 15 (18 hours), and is not detectable in RNA from a number of adult organs. To confirm that the oocyte cDNA encodes a gap junction channel, the protein was over expressed in *Xenopus* oocytes by injection of RNA synthesized in vitro. Pairs of RNA-injected oocytes formed many more time- and voltage-sensitive cell-cell channels than water-injected pairs.

CELLS OF THE EARLY AMPHIBIAN embryo are coupled by low-resistance pathways that are permeable to ions and small molecules. Gap junctions are thought to be responsible for this coupling. The precise roles of junctional coupling in the oocyte and early embryo are not known. Gap junctions couple follicular cells to the oocyte and may help control oocyte maturation (*1*). In embryonic tissues, it has been suggested that gap junctions transmit regulatory factors involved in the determination of regional patterns of differentiation (*2, 3*). Some similarity between gap junction proteins from amphibians and mammals is suggested by the report that antibodies generated against adult rat liver gap junctions cross-react with proteins in *Xenopus* blastulae (*2*). On the other hand, unlike mammalian gap junctions, voltage-clamp studies of pairs of amphibian blastomeres show that the steady-state junctional conductance is steeply voltage dependent (*4–6*).

To elucidate the molecular basis for the observed differences between amphibian and mammalian gap junction proteins, we have cloned and sequenced cDNA for a *Xenopus* gap junction protein. A cDNA library in λgt11 constructed from *Xenopus* oocytes was screened with a cDNA coding for rat connexin43 under low-stringency conditions (*7*). One positive clone, X01A, was obtained. The clone was subcloned into the plasmid vector Bluescript (Stratagene) and sequenced on both strands by means of the dideoxy chain termination method (*8*). The cDNA was 1301 nucleotides in length. The cDNA contained a single open reading frame of 1002 bases and portions of 5′ and 3′ untranslated regions. No polyadenylated tail or consensus polyadenylation signal was observed.

The DNA has been entered into the GenBank data base (access no. J03091). The open reading frame encodes a protein of 334 amino acids with a molecular mass of 38 kD. In accordance with the nomenclature suggested by Beyer *et al.* (*7*), this protein was named *Xenopus* connexin38. For brevity, we will refer to the predicted protein as connexin38. The sequence of connexin38 was compared with two rat gap junction proteins (*7, 9*), connexin32 and connexin43 (Fig. 1). Many amino acid residues are identical in all three proteins. The overall amino acid homology is 32% between connexins 38 and 32 and 41% between connexins 38 and 43 with regions of higher and lower homologies. Comparison of connexin38 with a rat lens gap junction protein, connexin46 (*10*), and a human liver gap junction protein (*11*) gives similar results. A search of the National Biomedical Research Foundation protein database and the GenBank and European Molecular Biology Laboratory DNA database identified no other sequences with significant homology.

A hydropathicity profile (*12*) of connexin38 predicts the same four potential transmembrane domains described by Beyer *et al.* (*7*). Unlike the other gap junction proteins, connexin38 was predicted to have a fifth hydrophobic domain close to the COOH-terminus. Lacking in the sequence for connexin38 is a transmembrane segment corresponding to the S4 region proposed to be involved in the voltage-dependent gating of Na^+ (*13*), dihydropyridine-sensitive Ca^{2+} (*14*), and fast, transient K^+ (*15*) channels.

An RNA blot analysis (Fig. 2) of *Xenopus* total RNA from different developmental stages demonstrated the presence of connexin38 RNA in eggs. The signal declined by early gastrula and was below the sensitivity of our assay by early neurula. Total RNA from adult tissues was also tested for the presence of connexin38 mRNA; a signal was detected in ovary but not in liver, heart, intestine, testes, and kidney. Connexin38 appears to have a distribution thus far detected only in the oocyte and early embryo. It does not have a distribution similar to connexin43 in the rat, which is found not only in the ovary, principally between ovari-

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XEN CXN38   1  MAGWELLKLLLDDVQEHSTLIGKVWLTVLFIFRIFILSVAGESVWTDEQSDFICNTQQPGCTNVCYDQAFPISHV  75
RAT CXN43   1  *GD*SA*GK***K**AY**AG*****S*******LL*GT*V**A*G****A*R********E*****KS******  75
RAT CXN32   1  *N-*TG*YT**SG*NR***A**R***S*I*****MVLV**A****G**K*S*I***L****NS****HF******  74

XEN CXN38  76  RYWVLQFLFVSTPTLTYLGHMVYLSKKEEKERQKENE----SRILVANE---AQTE----VY-SSATKKIRIQGP 138
RAT CXN43  76  *F****II***V***L**A*VF*VMR****LNK**E*LKVAQTDG*NV*MHLK*I*IKKFK*GIEEHG*VKMR*G 150
RAT CXN32  75  *L*S**LIL****A*LVAM*VAHQQHI*K*M------LRL---EGHGDPLHLE--*VKR-------H-*VH*S*T 130

XEN CXN38 139  LMCTYTTSVVFKSIFEAGFL-LG-QWYI-YGFVMSPIFVCERIPCKHKVECFVSRPMEKTIFIIFMLVVSLISLL 210
RAT CXN43 151  *LR**II*IL***V**VA**-*I-****-***SL*AVYT*K*D**P*Q*D**L***T*****I********V**A 222
RAT CXN32 131  *WW**VI****RLL***V*MYVFYLI*PG*AM-VRLV-K**AF**PNT*D******T***V*TV***AA*G*CII 203

XEN CXN38 211  LNLMELIHLSFKCFQHGIKEGATCPP-TGIPFNGAGNRMPPQE-YTNPPSSNQDIDLPAYNKMSGGHNWSSIQME 284
RAT CXN43 223  **II**FYVFF*GVKDRVKGRSDPYHA*TG*LSPSKDCGS*KYA*F*GC**PTAPLS*MSPPGYKLVTGDRNNSS 297
RAT CXN32 204  **VA*VVY*IIRACARRAQRRSNP*SRKGSGFGHRLSPEYKQN-EI*KLL*E**GS*KDILRRSP*TGAGLAEKS 277

XEN CXN38 285  QQVNGLVKPKCQCDCWSQSAISVVVSGAPGIISNMDAVKRNHQTSSKQQYV                        334
RAT CXN43 298  CRNYNKQASEQNWANY*AEQNRMGQA*STISN*HAQPFDFPDDNQNAKKVAAGHELQPLAIVDQRPSSRASSRAS 372
RAT CXN32 278  DRCSAC                                                                   283

RAT CXN43 373  SRPRPDDLEI                                                               382
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Fig. 1. Alignment of the amino acid sequence of *Xenopus* connexin38 (XEN CXN38) with rat connexin43 and connexin32 (RAT CXN43 and RAT CXN32). Identical residues are indicated by asterisks and gaps by dashes. Gaps are inserted manually to optimize the alignment.

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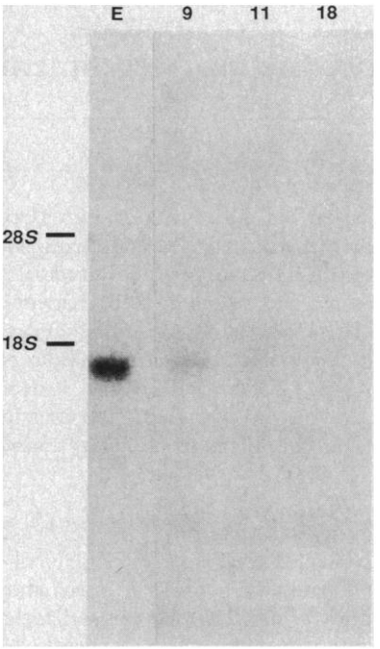

Fig. 2. Developmentally regulated expression of *Xenopus* connexin38. Total RNA was isolated at different developmental stages by the proteinase K–phenol extraction method (*20*). E, egg; 9, late blastula; 11, gastrula; 18, neurula. RNA samples from five embryos were separated by electrophoresis on 1% agarose gels containing formaldehyde, blotted to nylon membrane (Hybond-N, Amersham) and hybridized with labeled connexin38 cDNA in 0.75*M* Na_2PO_4 (*p*H 7.2), 5% SDS, and 100 μg of salmon sperm DNA per milliliter at 65°C. Filters were washed at 65°C in 0.03*M* Na_2PO_4 and 1% SDS and exposed overnight to XAR-5 film with intensifying screen at −80°C.

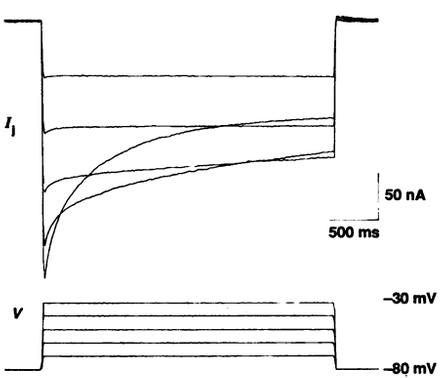

Fig. 3. Junctional currents (I_j) in oocyte pairs expressing connexin38. Pairs of *Xenopus* oocytes were voltage clamped with a double clamp procedure (*5*) in which one cell was held at a constant potential while voltage steps were applied to a second cell. *V* indicates the potential to which the second cell was stepped. Both cells were initially clamped at −80 mV. The complete XO1A cDNA was subcloned into the RNA expression vector, SP64T (*21*). The resulting construct contained connexin38 sequences in between 5′ and 3′ noncoding regions of *Xenopus* β globin. The presence of the β globin noncoding sequences substantially enhanced expression of connexin38 in oocytes. In vitro synthesis of RNA with unmethylated cap was carried out with SP6 polymerase (Promega Biotech) according to manufacturer's instructions. RNA (40 nl) at 0.5 μg/μl was pressure-injected into defolliculated oocytes. The follicular cell layer and vitelline membrane were removed by the procedures of Methfessel *et al.* (*22*). Electrical measurements were made 24 hours after injection and pairing.

an granulosa cells, but also in myocardium and kidney. Connexin38 also has a different distribution than a recently cloned *Xenopus* gap junction cDNA, termed XE11a (*16*). This cDNA predicts a 30-kD polypeptide, and its transcript appears at gastrulation in the developing embryo and is detectable in the adult in a pattern similar to the distribution of rat connexin32 in adult rat organs (*9*).

To test whether connexin38 can form functional channels, we used an oocyte cell-cell channel assay (*17*). Connexin38 RNA was synthesized in vitro and pressure-injected into oocytes. Vitelline membranes were manually removed and the cells were manipulated together to form pairs. Gap junction channel formation was measured by a dual-voltage clamping procedure (*4*). Six of six pairs of connexin38-injected cells obtained from a single ovary were strongly coupled with a mean junctional conductance (G_j) of 4.22 ± 2.56 μS. In contrast, water-injected controls obtained from the same ovary showed little or no coupling (G_j = 0.02 ± .05 μS, n = 8). Similar results were obtained in four other batches of oocytes (*18*).

To test whether the junctional conductance was voltage dependent, one member of the cell pair was clamped at a constant holding potential of −80 mV and the voltage of the second cell was stepped sequentially from −80 mV to voltages between −70 and −30 mV in 10 mV, 3-s increments. The junctional current (I_j) displayed a pronounced time and voltage dependence for voltage-clamp steps greater than 20 mV (Fig. 3). The time course of inactivation of I_j was a multiple exponential process, suggesting that inactivation is a complex phenomenon and may involve several component processes or more than one channel type. The time- and voltage-dependent properties of this conductance were qualitatively similar to those of the endogenous background channels. These properties differ from the properties of *Xenopus* blastomere pairs (*19*) in that the time course of the voltage-dependent decline in the conductance is more complex. This difference could be explained by either a change in the behavior of connexin38 channels in different cellular environments or the existence of additional endogenous gap junction proteins in the early amphibian embryo.

REFERENCES AND NOTES

1. C. L. Brown, H. S. Wiley, J. N. Dumont, *Science* **203**, 182 (1979).
2. A. E. Warner, S. C. Guthrie, N. B. Gilula, *Nature* **311**, 127 (1984).
3. S. Caveney, *Annu. Rev. Physiol.* **47**, 319 (1985).
4. D. C. Spray, A. L. Harris, M. V. L. Bennett, *Science* **204**, 432 (1979).
5. A. L. Harris, D. C. Spray, M. V. L. Bennett, *J. Gen. Physiol.* **77**, 95 (1981).
6. R. L. White *et al.*, *Am. J. Physiol.* **249**, C447 (1985); E. C. Reverdin and R. Weingart, *ibid.* **254**, C226 (1988).
7. E. C. Beyer, D. L. Paul, D. A. Goodenough, *J. Cell Biol.* **105**, 2621 (1987).
8. F. Sanger, S. Nicklen, A. R. Coulson, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463 (1977).
9. D. L. Paul, *J. Cell Biol.* **103**, 123 (1986).
10. E. C. Beyer, D. A. Goodenough, D. L. Paul, in *Gap Junctions*, E. L. Hertzberg and R. Johnson, Eds. (Liss, New York, 1988), pp. 167–174.
11. N. M. Kumar and N. B. Gilula, *J. Cell Biol.* **103**, 767 (1986).
12. J. Kyte and R. F. Doolittle, *J. Mol. Biol.* **157**, 105 (1980).
13. M. Noda *et al.*, *Nature* **312**, 121 (1984).
14. T. Tanabe *et al.*, *ibid.* **328**, 313 (1987).
15. D. M. Papazian, T. L. Schwartz, B. L. Tempel, Y. N. Jan, L. Y. Jan, *Science* **237**, 749 (1987).
16. R. L. Gimlich, N. M. Kumar, N. B. Gilula, *J. Cell Biol.* **107**, 1065 (1988).
17. C. Dahl, T. Miller, D. Paul, R. Voellmy, R. Warner, *Science* **236**, 1290 (1987).
18. Since the levels of connexin38-induced coupling and background endogenous coupling varied between frogs, experimental and control conductance values were always obtained from the same ovary and matched for stage of maturation. The conductance levels between pairs of water-injected controls were at or below the detection limits of our electronics (less than 50 nS) in four of five batches of oocytes. However, in one batch the background conductance level was somewhat higher (mean 280 nS). The conductances of the mRNA-injected oocytes in this batch were also higher (mean 15.2 μS). Dahl *et al.* (*17*) reported similar control values, ranging from 0 to 60 nS, except for one experiment with higher control conductances (180 nS).
19. D. C. Spray, A. L. Harris, M. V. L. Bennett, *J. Gen. Physiol.* **77**, 77 (1981).
20. P. A. Krieg and D. A. Melton, *EMBO J.* **13A**, 3463 (1985).
21. P. A. Krieg and D. A. Melton, *Nucleic Acids Res.* **12**, 7035 (1984).
22. C. Methfessel *et al.*, *Pfluegers Arch.* **407**, 577 (1986).

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