Sequence and Developmental Expression of mRNA Coding for a Gap Junction Protein in *Xenopus*

Robert L. Gimlich, Nalin M. Kumar, and Norton B. Gilula

Department of Molecular Biology, Research Institute of Scripps Clinic, La Jolla, California 92037

Abstract. Cloned complementary DNAs representing the complete coding sequence for an embryonic gap junction protein in the frog *Xenopus laevis* have been isolated and sequenced. The cDNAs hybridize with an RNA of 1.5 kb that is first detected in gastrulating embryos and accumulates throughout gastrulation and neurulation. By the tailbud stage, the highest abundance of the transcript is found in the region containing ventroposterior endoderm and the rudiment of the liver. In the adult, transcripts are present in the lungs, alimentary tract organs, and kidneys, but are not detected in the brain, heart, body wall and skeletal muscles, spleen, or ovary. The gene encoding this embryonic gap junction protein is present in only one or a few copies in the frog genome. In vitro translation of RNA synthesized from the cDNA template produces a 30-kD protein, as predicted by the coding sequence. This product has extensive sequence similarity to mammalian gap junction proteins in its putative transmembrane and extracellular domains, but has diverged substantially in two of its intracellular domains.

GAP junctions form an aqueous pathway connecting one cytoplasm to the next in a multicellular system (Gilula et al., 1972; Loewenstein, 1981). The channels that make up a vertebrate gap junction allow passage of ions and molecules <20 Å in diameter, and show weak charge selectivity (Simpson et al., 1977; Flagg-Newton et al., 1979; Schwartzmann et al., 1981; Brink and Dewey, 1980; Neyton and Trautmann, 1985). Thus, cells coupled to each other by gap junction channels form an electrical syncytium and potentially share a host of metabolic intermediates and effector molecules such as cyclic nucleotides and other "second messengers" (Pitts and Sims, 1977; Lawrence et al., 1978; Murray and Fletcher, 1984).

Gap junction-mediated intercellular coupling is widespread during embryonic development and pattern formation in many organisms. Regional variations in junctional communication can be correlated in some cases with specific morphogenetic behaviors and cell fate (reviewed by Caveney, 1985). In the frog *Xenopus laevis*, the pattern of gap junctional communication in early embryos seems to be related to the plane of bilateral symmetry. Animal hemisphere blastomeres near the future dorsal midline of the 32-cell embryo transfer the dye Lucifer Yellow to their neighbors more frequently than do cells flanking the ventral midline (Guthrie, 1984). This suggests that there are consistent dorsoventral differences in the number of functional gap junctional channels very early in development.

In *Xenopus* and other amphibian embryos, changes in the pattern of junctional coupling occur during several episodes of tissue morphogenesis and embryonic induction. Blackshaw and Warner (1976) found that ionic coupling between prospective myotomal cells in *Xenopus* disappears when the somites begin to segment and rotate, but then reappears. Electrical continuity has been observed between prospective chordamesoderm and neural plate cells of the newt embryo during the period of neural plate induction and regionalization (Ito and Ikematsu, 1980). While prospective epidermal and neural epithelial cells are well coupled across the neural plate border during neurulation in the axolotl, this becomes a communication boundary at about the time the neural folds meet and fuse (Warner, 1973). Finally, junctional coupling among progenitor cells of the central nervous system in *Xenopus* embryos can be reduced by microinjection of antibodies raised against the predominant protein of rat liver gap junctions (Warner et al., 1984). In these embryos, abnormalities in development of the brain, eye, and other anterior structures are common.

Taken together, these results suggest that cell-cell interactions crucial for normal development in amphibia involve regulated diffusion of ions or small molecules via gap junctions. Modulation of gap junctional communication during embryonic development may reflect regulated conductance at existing junctions or alterations in the number of junctions in the plasma membranes. Junctional conductance is affected by intracellular events such as membrane depolarization, calcium and proton fluxes, and changes in intracellular second messenger activities (reviewed by Spray and Bennett, 1985). Gap junction number may be regulated at many levels – from the control of gene transcription to rates of protein modification and degradation. Finally, structurally distinct

Dr. Gimlich's present address is Department of Zoology, Oregon State University, Corvallis, OR 97331.

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gap junction proteins may be present during embryogenesis, as they are in diverse adult organs (Hertzberg et al., 1982; Gros et al., 1983; Kumar and Gilula, 1986; Beyer et al., 1987; see also Kistler et al., 1988; Nicholson et al., 1987).

To fully understand the developmental role of gap junctions, it is essential to determine their number and distribution in the embryo, their chemical composition and structure, and the mechanisms which regulate their activity. This paper describes a first step in this direction using a system especially suited for embryological study, the *Xenopus* embryo. Complementary DNAs that contain the complete coding sequence for a gap junction protein in *Xenopus* have been cloned and sequenced. In addition, the pattern of accumulation of mRNA encoding this protein has been determined.

Materials and Methods

Collection, Culture, and Staging of Embryos

Adult *Xenopus laevis* were maintained at 22°C on a 12-h light/dark cycle and a diet of chopped beef liver and Purina Trout Chow. Ovulation was induced by injection of 400-700 IU of human chorionic gonadatropin (Sigma Chemical Co., St. Louis, MO) into the dorsal lymph sac, and stripped eggs were fertilized in vitro with minced testis. Fertilized eggs were dejellied in a solution of 0.1 M NaCl, 25% (wt/vol) l-cysteine hydrochloride (Sigma Chemical Co.), pH 7.9. Embryos were cultured at 18-22°C in 33% modified Ringers solution (33% MR; MR is 0.1 M NaCl, 0.002 M KCl, 0.001 M $MgCl_2$, 0.002 M $CaCl_2$), and staged according to the normal table of *Xenopus* development (Nieuwkoop and Faber, 1975). Oocytes were staged according to Dumont (1972). Embryos at stage 26 were dissected with watchmaker's forceps and a hair-loop in a bath of 33% MR on an agarose substratum.

Extraction of Nucleic Acids from Embryos and Tissues

RNA was extracted from whole embryos or microdissected embryonic parts by the method of Auffray and Rougeon (1980) as modified by Mohun et al. (1984). RNA was prepared from adult tissues by homogenization in a 4.0 M guanidinium thiocyanate buffer (Chirgwin et al., 1979), followed by sedimentation through a layer of 5.7 M cesium chloride (Glisin et al., 1974). Polyadenylated RNA was selected by chromatography on oligo-dT cellulose (Aviv and Leder, 1972).

High molecular weight genomic DNA was prepared by pulverizing a freshly dissected frog liver in liquid nitrogen and homogenizing in 0.01 M Tris-Cl (pH 7.4), 0.5 M NaCl, 0.025 M EDTA, 1.0% SDS, 0.1 mg/ml proteinase K. DNA was extracted sequentially with phenol/chloroform (1:1), chloroform, and ether, and spooled out from 2.5 vol of cold 100% ethanol.

Screening of cDNA Clone Libraries

A cDNA library constructed in lambda gt10 (Huynh et al., 1985) from *Xenopus* liver polyadenylated RNA was kindly supplied by Dr. David Shapiro (University of Chicago). Approximately 5×10^5 phage from this library were screened by hybridization at low stringency (45% formamide, 5× SSC, 37°C, with washing in 2× SSC, 37°C) with a 997-bp Eco RI-Stu I fragment of the human liver gap junction cDNA clone decribed by Kumar and Gilula (1986). A single hybridizing plaque was isolated. The 426-bp Eco RI insert of *Xenopus* cDNA in this phage, designated XL1, was subcloned in M13mp19 for DNA sequencing. The cDNA insert was purified from the replicative form of M13 for further screening. A lambda gt10 cDNA library made with stage-11 embryo polyadenylated RNA was kindly supplied by Dr. Douglas Melton (Harvard University). Hybridization screening of this library was done in 50% formamide, 5× SSC at 37°C, with washing in 0.2× SSC at 45°C. Hybridization probes were synthesized from double-stranded DNA using the large fragment of DNA polymerase I and random hexameric oligonucleotides (Pharmacia, Piscataway, NJ) as primers (Feinberg and Vogelstein, 1983).

DNA Sequence Analysis

cDNA fragments were excised from the bacteriophage vector and inserted into the Eco RI site of M13mp19 for DNA sequencing by the chain termination method as modified by Biggens et al. (1983). Sequencing reactions at the fragment termini were primed with the 17-nucleotide universal sequencing primer (New England Biolabs, Beverly, MA), and further sequencing reactions were primed with synthetic oligonucleotides complementary to *Xenopus* cDNA sequences. DNA oligonucleotides were synthesized with an Applied Biosystems synthesizer (model 380A; Applied Biosystems, Inc., Foster City, CA). DNA sequences were compiled and analyzed using PCGene software (Intelligenetics, Palo Alto, CA).

RNA and DNA Blot Analyses

RNAs were separated by electrophoresis through 1% agarose gels containing 0.66 M formaldehyde and transferred to Biodyne nylon filters (Pall Corp., Glen Cove, NY) for hybridization with radiolabeled fragments of cloned *Xenopus* cDNA. A probe for *Xenopus* actin mRNAs was obtained from Dr. T. Sargent (National Institutes of Health). This was a 1.1-kb cDNA from a library constructed by Dworkin and Dawid (1980), which represents a muscle actin mRNA. When hybridized at low stringency with *Xenopus* RNAs, this probe detected all of the actin isoform mRNAs (Sargent et al., 1986). Mitochondrial RNAs were detected using a probe consisting of the cloned 17.4-kb *Xenopus* mitochondrial genome (Chase and Dawid, 1972). Genomic DNA was digested with several restriction endonucleases and fractionated on 0.7% agarose gels for transfer to nylon filters and hybridization with cDNA probes. Low stringency hybridizations with filter-bound nucleic acids were done in 40% formamide, 5× SSC, at 37°C, followed by washing in 2× SSC at 37°C. High stringency hybridizations were done at 37°C in 50% formamide, 5× SSC, washing in 0.2× SSC at 60°C.

Nuclease Protection Analysis

RNA was purified from embryos at various developmental stages and hybridized in solution with single-stranded DNA probes. The probes were synthesized from a (+)-strand M13mp19 subclone using the large fragment of DNA polymerase I as described (Messing, 1983), in the presence of [α-^{32}P]dCTP (800-1,200 Ci/mmol; Amersham Corp., Arlington Heights, IL). Synthesis was primed with the "−40", 17-mer oligonucleotide sequencing primer (New England Biolabs). The double-stranded extension product was digested with restriction endonucleases (New England Biolabs) Acc I (base 590) or Hinf I (base 745). The resulting antisense strand probes were purified by electrophoresis in alkaline agarose gels (Burke, 1984). After hybridization with RNA (250-625 μg/ml) in 80% formamide, 0.4 M NaCl at 55°C, residual single-stranded probe was digested at 37°C with S1 nuclease (Bethesda Research Laboratories, Gaithersburg, MD) as described (Berk and Sharp, 1977). DNA fragments protected by annealing with RNA were separated by electrophoresis in 6% polyacrylamide gels containing 6 M urea and detected by autoradiography at −70°C using Kodak XAR-5 film with an intensifying screen.

Absolute transcript numbers were estimated by probe excess hybridization, or titration (Lev et al., 1980), with varying amounts of unfractionated or polyadenylated RNA. The total RNA mass in each reaction was adjusted to 30 μg by addition of yeast tRNA. Hybridization and S1 nuclease digestion were followed by phenol/chloroform (1:1) extraction and ethanol precipitation. Radioactive hybrids were bound to DE81 filters (Schleicher & Schuell, Inc., Keene, NH), washed in 0.5 M Na_2HPO_4, and quantitated by scintillation counting. Background nuclease-resistant radioactivity was determined using parallel reactions with 30 μg yeast tRNA. Transcript abundance was calculated as described by Scheller et al. (1978). The accuracy of this method was checked using control hybridizations with in vitro-synthesized RNA (see below).

In Vitro Translation of Xenopus Gap Junction Polypeptides

An Eco RI fragment from the stage-11 embryonic gap junction cDNA clone was inserted into the vector pT7/T3-18 (Bethesda Research Laboratories). Coding RNA was transcribed in vitro using T3 bacteriophage RNA polymerase after linearizing the plasmid with Pvu II. Antisense RNA was transcribed using T7 polymerase after linearizing with Hind III. Bacteriophage RNA polymerases were purchased from Bethesda Research Laboratories, and transcription reactions were as described by Melton et al. (1984).

In vitro translation was performed using ∼1 μg of synthetic RNA in 50 μl of rabbit reticulocyte lysate (Bethesda Research Laboratories), containing [^{35}S]methionine (Amersham Corp.). Translation reactions were terminated by addition of SDS-containing gel loading buffer. Translation prod-

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      5'
X   GCTTTCCATTTCAGCTACCCAGTGATTGGAACACAGGAGAGCAGCTAACACATCTAACACA 61

X   ATG AAT TGG GCA GGA TTA TAC GCC ATA CTG AGT GGC GTG AAC CGC CAC TCC ACC TCA ATC GGA CGC ATA TGG CTC TCT GTG GTC TTC ATC 151
H   ATG AAC TGG ACA GGT TTG TAC ACC TTG CTC AGT GGC GTG AAC CGG CAT TCT ACT GCC ATT GGC CGA GTA TGG CTC TCG GTC ATC TTC ATC

X    M   N   W   A   G   L   Y   A   I   L   S   G   V   N   R   H   S   T   S   I   G   R   I   W   L   S   V   V   F   I   30
H    M   N   W   T   G   L   Y   T   L   L   S   G   V   N   R   H   S   T   A   I   G   R   V   W   L   S   V   I   F   I

X   TTC CGT ATC ATG GTG CTT GTG GCG GCT GCA GAA AGC GTA TGG GGG GAT GAG AAG TCC GCG TTT ACA TGC AAC ACA CAA CAG CCC GGT TGC 241
H   TTC AGA ATC ATG GTG CTG GTG GTG GCT GCA GAG AGT GTG TGG GGT GAT GAG AAA TCT TCC TTC ATC TGC AAC ACA CTC CAG CCT GGC TGC

X    F   R   I   M   V   L   V   A   A   A   E   S   V   W   G   D   E   K   S   A   F   T   C   N   T   Q   Q   P   G   C   60
H    F   R   I   M   V   L   V   V   A   A   E   S   V   W   G   D   E   K   S   S   F   I   C   N   T   L   Q   P   G   C

X   AAC AGT GTA TGC TAT GAT CAC TTC TTC CCC ATC TCA CAT ATC CGT CTG TGG GCC CTC CAG CTA ATC ATT GTA TCC ACA CCT GCC CTT CTG 331
H   AAC AGC GTT TGC TAT GAC CAA TTC TTC CCC ATC TCC CAT GTG CGG CTG TGG TCC CTG CAG CTC ATC CTA GTT TCC ACC CCA GCT CTC CTC

X    N   S   V   C   Y   D   H   F   F   P   I   S   H   I   R   L   W   A   L   Q   L   I   I   V   S   T   P   A   L   L   90
H    N   S   V   C   Y   D   Q   F   F   P   I   S   H   V   R   L   W   S   L   Q   L   I   L   V   S   T   P   A   L   L

X   GTG GCC ATG CAC GTG GCT CAT CTA CAG CAC CAA GAG AAG AAG GAG CTA CGT TTG TCC GGC CAT GTT AAG GAC CAA GAG CTG GCA GAA GTG 421
H   GTG GCC ATG CAC GTG GCT CAC CAG CAA CAC ATA GAG AAG AAA ATG CTA CGG CTT GAG GGC CAT GGG GAC CCC CTA CAC CTG GAG GAG GTG

X    V   A   M   H   V   A   H   L   Q   H   Q   E   K   K   E   L   R   L   S   G   H   V   K   D   Q   E   L   A   E   V  120
H    V   A   M   H   V   A   H   Q   Q   H   I   E   K   K   M   L   R   L   E   G   H   G   D   P   L   H   L   E   E   V

X   AAG AAA CAT AAA GTC AAG ATT TCC GGC ACT TTG TGG TGG ACC TAC ATC TCT AGT GTT TTC TTC AGA ATC ATA TTC GAG GCA GCC TTT ATG 511
H   AAG AGG CAC AAG GTC CAC ATC TCA GGG ACA CTG TGG TGG ACC TAT GTC ATC AGC GTG GTG TTC CGG CTG TTG TTT GAG GCC GTC TTC ATG

X    K   K   H   K   V   K   I   S   G   T   L   W   W   T   Y   I   S   S   V   F   F   R   I   I   F   E   A   A   F   M  150
H    K   R   H   K   V   H   I   S   G   T   L   W   W   T   Y   V   I   S   V   V   F   R   L   L   F   E   A   V   F   M

X   TAC ATC TTC TAC CTC ATC TAC CCT GGT TAC TCC ATG ATC CGA CTT GTT AAG TGC GAT GCC TAT CCC TGC CCC AAC ACT GTA GAC TGT TTC 601
H   TAT GTC TTT TAT CTG CTC TAC CCT GGC TAT GCC ATG GTG CGG CTG GTC AAG TGC GAC GTC TAC CCC TGC CCC AAC ACA GTG GAC TGC TTC

X    Y   I   F   Y   L   I   Y   P   G   Y   S   M   I   R   L   V   K   C   D   A   Y   P   C   P   N   T   V   D   C   F  180
H    Y   V   F   Y   L   L   Y   P   G   Y   A   M   V   R   L   V   K   C   D   V   Y   P ^ C   P   N   T   V   D   C   F

X   GTT TCT CGT CCC ACG GAG AAG ACC ATA TTC ACC GTC TTC ATG CTC GTC GCC TCT GGG GTC TGC ATC GTT CTG AAT GTT GCT GAA GTA TTC 691
H   GTG TCC CGC CCC ACC GAG AAA ACC GTC TTC ACC GTC TTC ATG CTA GCT GCC TCT GGC ATC TGC ATC ATC CTC AAT GTG GCC GAG GTG GTG

X    V   S   R   P   T   E   K   T   I   F   T   V   F   M   L   V   A   S   G   V   C   I   V   L   N   V   A   E   V   F  210
H    V   S   R   P   T   E   K   T   V   F   T   V   F   M   L   A   A   S   G   I   C   I   I   L   N   V   A   E   V   V

X   TTC CTG ATT GCC CAG GCC TGC ACC AGA AGA GCC --- CGC CGC --- --- --- --- --- CAC AGG GAT TCT GGC --- AGC --- --- ATC AGC 754
H   TAC CTC ATC ATC CGG GCC TGT GCC CGC CGA GCC CAG CGC CGC TCC AAT CCA CCT TCC CGC AAG GGC TCG GGC TTC GGC CAC CGC CTC TCA

X    F   L   I   A   Q   A   C   T   R   R   A   -   R   R   -   -   -   -   -   H   R   D   S   G   -   S   -   -   I   S  231
H    Y   L   I   I   R   A   C   A   R   R   A   Q   R   R   S   N   P   P   S   R   Y   G   S   G   F   G   H   R   L   S

X   AAA GAG CAT CAA CAG AAT GAA ATG AAC --- TTG CTG --- ATC ACG GGA GGC AGT --- --- --- ATT ATT AAA CGG TCC CCT --- --- --- 820
H   CCT GAA TAC AAG CAG AAT GAG ATC AAC AAG CTG CTG AGT GAG CAG GAT GGC TCC CTG AAA GAC ATA CTG CGC CGC AGC CCT GGC ACC GGG

X    K   E   H   Q   Q   N   E   M   N   -   L   L   -   I   T   G   G   S   -   -   -   I   I   K   R   S   P   -   -   -  253
H    P   E   Y   K   Q   N   E   I   N   K   L   L   S   E   T   D   G   S   L   K   D   I   L   R   R   S   P   G   T   G

X   GCG GGG CAG --- GAG AAA GGA GAT CAC TCT TCT ACC TCC TAA AGGATAAGGCTTCTAACTCTCACGGCTCCCTGGTGGGACATGACGGAGAGGGCAGCCCTGGAG 922
H   GCT GGG CTG GCT GAA AAG AGC GAC CGC TGC TCG GCC TGC TGA

X    A   G   Q   -   E   K   G   D   H   C   S   T   S  ***                                                                 265
H    A   G   L   A   E   K   S   D   R   C   S   A   C  ***

                                                                                                           3'
X   ATCATACAGCTACAAAAGCAAAGATGAGATATTGGGGTTAGTTACAAGACTTGAACAGGAGCAGTGTAAACGAAACGTGGC^AAGAAAAGGAATAGAGAAGCAAAAGAAAA      1032
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Figure 1. Nucleotide sequence of the *Xenopus laevis* embryonic gap junction cDNA. The complete sequence of the *X*E11a cDNA is presented (lines designated *X*) together with the amino acid sequence deduced from its longest open reading frame (in single letter code). Numbering at right refers to the *Xenopus* sequences. The human liver gap junction coding sequence and translation product is shown for comparison (lines designated *H*). Dashes indicate gaps introduced into the frog sequence for optimal alignment with the human codons. Underlined sequences indicate the positions of the four putative transmembrane helices (Milks et al., 1988). Arrowheads identify the 5′ and 3′ ends of the *Xenopus* liver partial cDNA *XL*1. The three overlined cytosine residues (817–819) were missing in the *XL*1 cDNA, leading to the absence of proline residue 253. Both strands of each cDNA were completely sequenced as described in Materials and Methods.

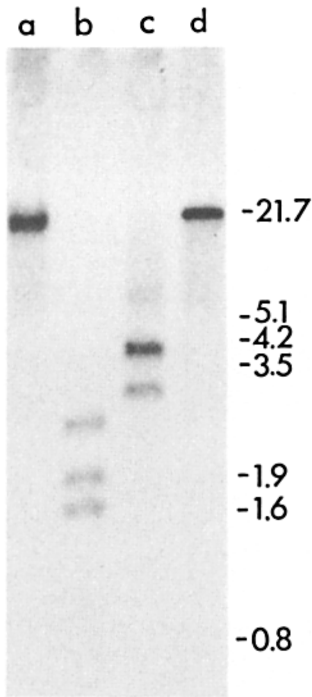

Figure 2. Southern analysis of genomic DNA. Frog liver DNA was digested to completion with the following restriction endonucleases: (*a*) Bam HI, (*b*) Dde I, (*c*) Pst I, and (*d*) Eco RI. Separated fragments were immobilized on nylon and hybridized with an *XE*11a DNA probe. Blots were washed at high stringency, and exposed to x-ray film for 1 wk. Size markers (in kilobases) are lambda DNA fragments of an Eco RI, Hind III digest.

ucts were separated on 12.5% polyacrylamide gels with 4% polyacrylamide stacking gels (Dreyfuss et al., 1984). Gels were permeated with Amplify (Amersham Corp.), dried, and exposed at −70°C to Kodak XAR-5 film with intensifying screens.

Results

Cloning of Xenopus Gap Junction cDNAs

The antigenic properties of gap junctions have been highly conserved in the evolution of deuterostomes (Hertzberg and Skibbens, 1984; Warner et al., 1984; Fraser et al., 1987). The complete primary structure of the 32-kD protein subunit of the mammalian liver gap junction has been deduced from the sequences of cloned cDNAs (Kumar and Gilula, 1986; Paul, 1986). Since embryos and adult tissues of *Xenopus laevis* contain proteins antigenically related to the rat liver junction protein (Warner et al., 1984; Green, C. R., unpublished results), it was anticipated that frog and mammalian liver would contain homologous gap junction mRNAs. To recover related mRNA sequences, *Xenopus* cDNA libraries were screened by hybridization at low stringency with the human liver gap junction cDNA.

A 997-bp restriction fragment of the human cDNA (Kumar and Gilula, 1986) containing the 5′ untranslated region, the 849-bp protein coding sequence, and 85 bp of the 3′ untranslated region was isolated. This fragment was labeled to high specific activity and used to screen a frog liver cDNA library in lambda gt10. In a screen of 500,000 recombinant phage, a single hybridizing plaque was obtained. After plaque purification, the phage genome was found to contain a 426-bp cDNA insert which hybridized to the human gap junction probe. The insert was purified and subcloned in M13mp19 for DNA sequencing. It contained a 282-bp open reading frame sharing 67 of 111 codons with the sequence encoding the carboxyl-terminal portion of the human liver

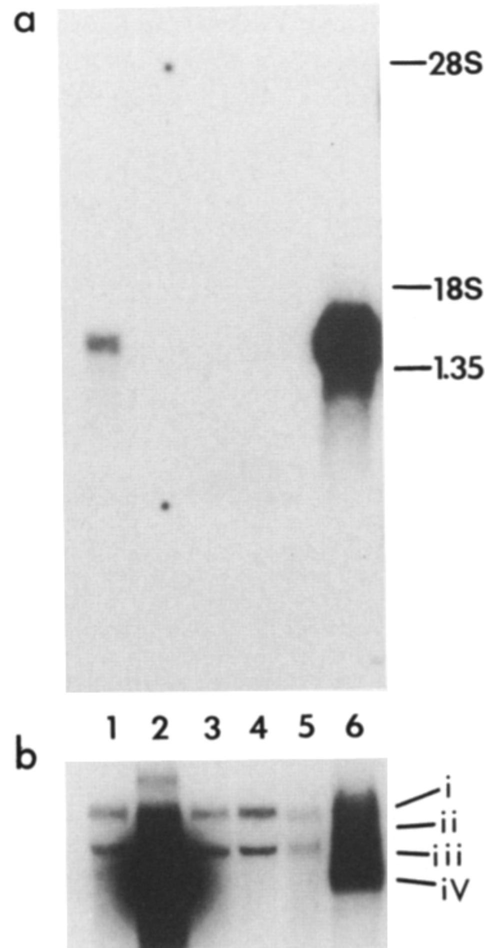

Figure 3. Northern blot analysis. (*a*) Gel blots with 2.5 μg of polyadenylated RNA from *Xenopus* adult tissues and embryos were probed with the radioactive *XE*11a cDNA insert and washed at high stringency. Lanes *1–6* contain RNA from (*1*) liver, (*2*) heart, (*3*) ovary, (*4*) stage VI oocytes, (*5*) stage 10 gastrulae, and (*6*) stage 15–16 neurulae. Size standards are ethidium bromide stained ribosomal RNAs and an RNA standard of 1.35 kilobases. (*b*) As a control for RNA integrity and transfer efficiency, the filter was washed and probed for actin mRNAs using a skeletal muscle actin cDNA probe (Sargent et al., 1986). Lanes show the expected relative abundances of cytoskeletal (bands *i* and *ii*) and muscle (bands *iii* and *iv*) actin isoform mRNAs.

gap junction protein. This partial cDNA clone was designated *XL*1 (Fig. 1).

The *XL*1 insert was excised and purified from the replicative form of M13mp19 and labeled to high specific activity for use as a hybridization probe. A screen of ~150,000 recombinants from a stage-11 gastrula cDNA library yielded a single hybridizing plaque. The 1,032-bp insert in this phage was subcloned in M13mp19 and both strands were sequenced as described in Materials and Methods. The complete sequence of this cDNA, designated *XE*11a, is shown in Fig. 1.[1] A 795-bp open reading frame encodes a polypeptide with striking similarity to mammalian liver gap junction pro-

1. These sequence data have been submitted to the EMBL/GenBank Data Libraries under the accession number Y00791.

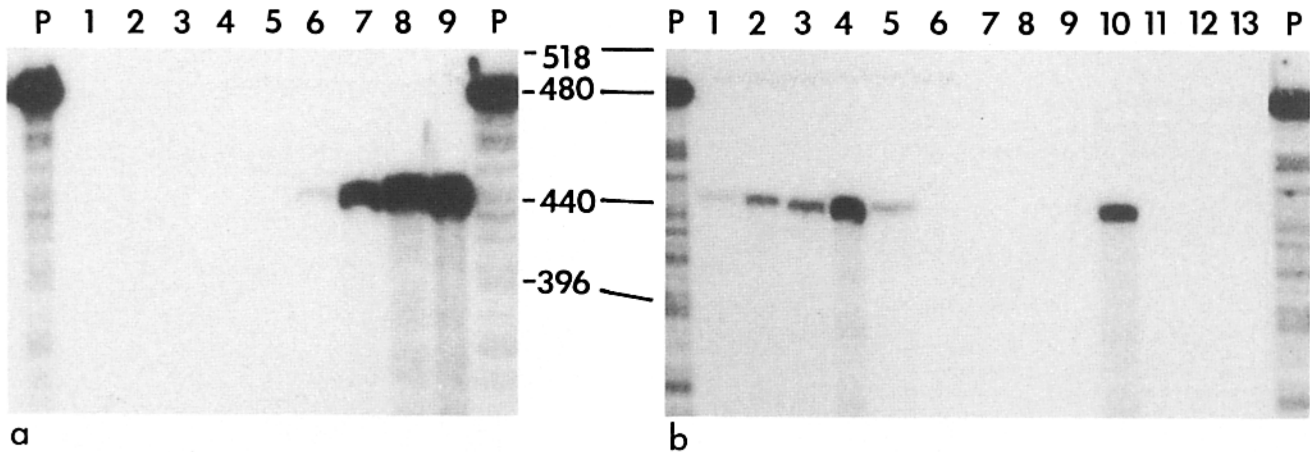

Figure 4. Nuclease protection analysis. A 480 nucleotide single-stranded DNA probe synthesized from *X*E11a was annealed to completion with 25 µg of each RNA. A fragment of 440 nucleotides is protected by hybridization from S1 nuclease digestion. *a* shows the accumulation of transcripts during embryogenesis. *P*, probe; RNA from (*1*) yeast, (*2*) *Xenopus* ovary, (*3*) fertilized eggs, (*4*) stage 8, (*5*) stage 10, (*6*) stage 12, (*7*) stage 14, (*8*) stage 19, and (*9*) stage 25. Northern analysis of these RNAs with a mitochondrial genomic DNA probe showed approximately equivalent recovery of mitochondrial ribosomal RNAs and mRNAs (data not shown). *b* shows hybridizations to RNA extracted from (*1*) *Xenopus* lung, (*2* and *3*) liver, two independent preparations, (*4*) intestine, (*5*) stomach, (*6*) spleen, (*7*) body wall muscle, (*8*) larynx, (*9*) thigh muscle, (*10*) kidney, (*11*) heart, (*12*) brain, and (*13*) yeast. Abundant intact actin mRNAs were detected in each of the *Xenopus* samples by Northern analysis (data not shown). Markers at 518 and 396 nucleotides are Hinf I fragments of pGEM1 DNA.

teins. When the predicted frog and human amino acid sequences are aligned for optimal fit, they show ~71% identity. Of the 272 nucleotide alterations between human liver and frog embryonic coding sequences, 115 are conservative, leaving amino acid coding unchanged.

Like the mammalian liver gap junction proteins (Kumar and Gilula, 1986; Paul, 1986), the protein encoded by the *X*E11a sequence contains four hydrophobic domains capable of spanning the lipid bilayer (Kyte and Doolittle, 1980). The location of putative transmembrane domains indicated in Fig. 1 is based on experimental and theoretical considerations detailed by Milks et al. (1988). Each sequence is rich in charged and polar amino acids at residues 100–130 and in the carboxy-terminal 40–60 residues. Only 45 of the first 223 amino acids differ between frog and human sequences; similarities in the carboxy-terminal region are more limited. There are 20 positions in the two sequences at which codon changes produce a change in charge at neutral pH; however, the net charge difference between the predicted frog and human proteins is 0. Based on these sequence similarities, the product of the *X*E11a coding sequence was tentatively identified as a *Xenopus* embryonic gap junction protein.

Genomic Sequences Encoding Embryonic Gap Junctions

To estimate the number of genes encoding this putative gap junction protein, chromosomal DNA was prepared from the liver of an individual frog and digested with several restriction endonucleases. The pattern of hybridization of the *X*E11a probe to separated restriction fragments is shown in Fig. 2. Digestion of genomic DNA with Bam HI or Eco RI, neither of which cleaves the cDNA, produced a single fragment of 16–20 kb with complementarity to the cDNA probe. The enzymes Pst I and Dde I both recognize single sites in the cDNA. Digestion with Pst I generated two fragments which hybridized with the probe. Three Dde I fragments in genomic DNA bound the *X*E11a probe. This could indicate the presence of an intron containing a Dde I site, or the occurrence of sequence polymorphism at this locus. Low stringency hybridization of *X*E11a with genomic DNA did not reveal any additional hybridizing fragments (data not shown). These results suggest that the *X*E11a genomic sequence is unique or exists in highly conserved copies.

Abundance of Gap Junction mRNAs in Embryos and Frog Tissues

Polyadenylated RNA was purified from adult tissues and from embryos at various developmental stages. RNA gel blots probed with the radiolabeled *X*E11a fragment revealed hybridizing transcripts of ~1.5 kb in polyadenylated RNA from adult liver and neurula stage embryos (Fig. 3). The transcripts were not detected in polyadenylated RNA from the heart or the ovary, nor in stage-VI oocytes or early gastrula stage embryos. These RNA samples contained abundant intact actin mRNAs, showing that they were intact.

A more detailed determination of the abundance of transcripts complementary to *X*E11a is provided by S1 nuclease protection assays. A radiolabeled single-stranded DNA probe complementary to bases 590–1,030 of the *X*E11a coding sequence was annealed in solution with unfractionated RNA from adult frog tissues and embryos. After digestion with S1 nuclease, protected probe fragments were resolved on sequencing gels. Transcripts which hybridize to this probe were not detected in total RNA (Fig. 4 *a*) or polyadenylated RNA (data not shown) from ovaries or stage VI oocytes, but accumulated between stages 10 and 12, the early to middle gastrula stages. Accumulation continued until at least stage 25, after the end of neurulation. In adults, transcripts were detected in RNA from the lungs, liver, intestine, stomach,

Table I. Titration of Excess XE1 1a Probe with Liver and Neurula RNAs

RNA assayed	Experiment	Mass probe protected per μg RNA	Mass transcript* per μg RNA
		pg	*pg/μg*
Liver poly(A)‡	A‡	0.33	
	B§	0.52	1.35 ± 0.42
Neurula total RNA	A	0.20	
	B	0.18	0.60 ± 0.04

* Transcript mass was calculated as probe mass times the length ratio of transcript to protected probe (1,500:440).
‡ Probe specific activity was 5 × 10^8 cpm/μg. Samples of 0.5, 1.0, 2.0, 3.0, or 5.0 μg polyadenylated RNA were hybridized with 5 × 10^4 cpm, or 100 pg probe, per assay. Linear correlation coefficient >0.97.
§ Probe specific activity was 1 × 10^9 cpm/μg. Samples of 5.0, 10.0, 20.0, or 30.0 μg of stage-19 total RNA were assayed, using 1 × 105 cpm, or 100 pg probe. Linear correlation coefficient >0.96.

and mesonephric kidney, but not in spleen, body wall, thigh muscle, heart, or brain (Fig. 4 *b*). These results confirm and extend the results of Northern blot analysis of polyadenylated RNAs.

Transcript abundance in several of the RNA samples was quantitatively determined using probe-excess titration with varying amounts of RNA (Table I). Total RNA from the late neurula stage (stage 19) contained 2–5 × 10^6 copies of the putative junction mRNA per embryo equivalent (i.e., per 4 μg RNA). By comparison, the abundance of α-cardiac actin mRNA was estimated to be 10^7–10^8 copies per embryo at this stage (Mohun et al., 1984). Titration analysis also showed that transcripts complementary to *X*E11a were at least 10-fold rarer in liver than in neurulae. Putative gap junction mRNA comprised <0.001% of the mass of polyadenylated RNA in liver. Assuming that 1-1.5% of the RNA mass is polyadenylated (Sagata et al., 1980), the transcripts accounted for as much as 0.01% of the poly(A)-containing RNA from the stage-19 embryo. These quantitative results also suggested that the transcript abundance in the ovary and oocytes was at most 10^{-5}% of the poly(A)-containing maternal RNA, or 0.01 times the abundance found in the liver.

Regional Abundance of Gap Junction RNAs

The distribution of putative gap junction mRNAs in the embryo was also assessed by hybridization analysis. Tadpoles at stage 26 were dissected into anterior and posterior portions by cutting just caudad to the otic vesicle and heart rudiment. Dorsal and ventral subfragments were then made by cutting through the cement gland and branchial region, along the flank just ventral to the pronephric duct rudiment, and through the closed blastopore or proctodaeum, as shown in Fig. 5 *a*. Equivalent amounts of total RNA from each region were assayed by nuclease protection (Fig. 5 *b*). Complementary RNA was detected in several of the fragments, but was most abundant in the ventral pieces. These contain ventrolateral epidermis and mesoderm, and the rudiments of the heart and endodermal structures (Nieuwkoop and Faber, 1975). Within the ventral portion of the embryo, most of the putative gap junction mRNA was found at, or posterior to, the position of the liver diverticulum.

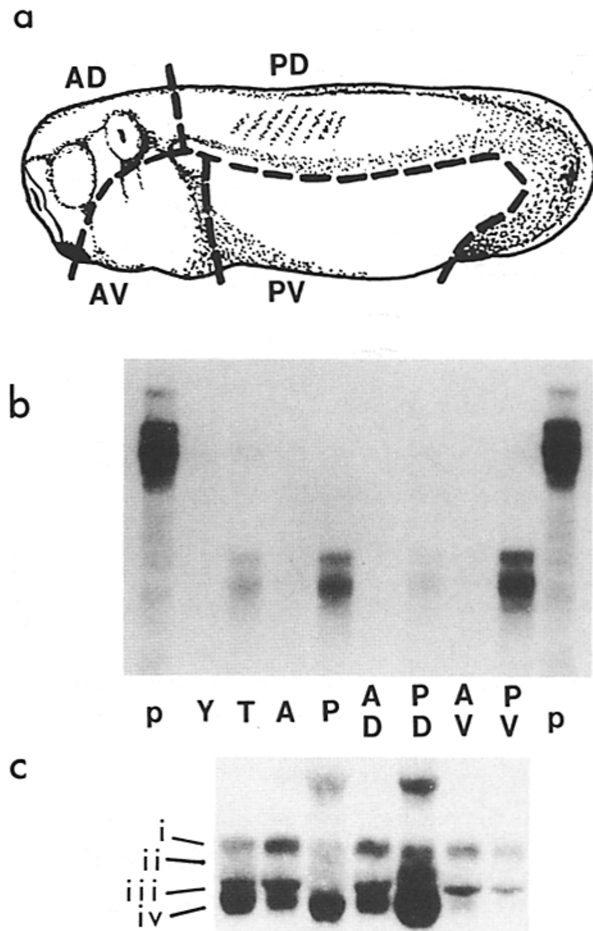

Figure 5. Distribution of gap junction transcripts in early tailbud stage embryos. (*a*) Embryos at stage 25 were dissected as shown (*heavy lines*). Fragments included all germ layer derivatives in each region. (*b*) RNA (10 μg) from each portion was assayed by S1 nuclease protection using a single stranded *X*E11a probe (*p*). Lanes: (*T*) total RNA from intact tadpoles, from (*A*) anterior fragments (*AD* plus *AV*), (*P*) posterior fragments (*PD* plus *PV*); (*AD*) dorsal head, (*PD*) dorsal trunk and tail, (*AV*) anteroventral, and (*PV*) posteroventral fragments. The probe, generated by Hinf I digestion after primer extension, migrated as three similarly sized single-stranded fragments of ∼340 nucleotides. Fragments of ∼300 nucleotides were protected by hybridization from S1 nuclease digestion. (*c*) Northern blot analysis of actin mRNA content in the same RNA samples. Actin isoform RNAs are designated as in Fig. 4 b.

In Vitro Translation of the Embryonic Gap Junction cDNA

The *Xenopus* cDNA sequence described above potentially encodes a protein of 30,122 D. In vitro translation of synthetic RNA transcribed from the *X*E11a insert is shown in Fig. 6. Addition of coding strand RNA transcribed in vitro (Melton et al., 1984) to a rabbit reticulocyte lysate stimulated synthesis of a major product migrating as a 30-kD protein in polyacrylamide gels. In contrast, synthetic mRNA for the human 32-kD liver gap junction protein (Kumar and Gilula, 1986) directed synthesis of a product migrating discernably slower than the frog protein. Control translations with in vitro transcribed noncoding strand RNA from the *X*E11a clone produced no labeled proteins besides the endogenous

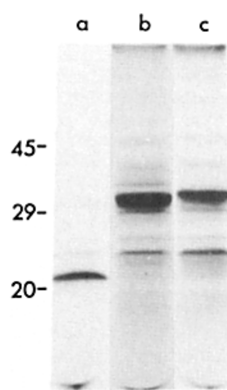

Figure 6. In vitro translation of synthetic gap junction mRNA. *Xenopus* or human liver gap junction cDNAs were cloned in the vector pT3/T7-18 and coding strand RNA was synthesized and translated in vitro as described in Materials and Methods. Translation products labeled with [35S]methionine were separated by PAGE and detected by fluorography. Translation products resulting from addition of (*a*) no exogeneous RNA, (*b*) *X*El1a coding strand RNA, and (*c*) human liver gap junction protein coding strand are shown. Addition of noncoding strand RNAs consistently yielded a pattern similar to that in lane *a* (data not shown). The major bands above 29 kD represent full-length translation products, while minor bands at 23 kD and below are attributable to premature termination of translation or proteolysis. Size markers are soybean trypsin inhibitor (20.1 kD), bovine carbonic anhydrase (29 kD), and egg albumin (45 kD).

translation products (data not shown). Thus, the cloned *X*El1a sequence is capable of directing expression of the predicted 30-kD protein.

Discussion

The *Xenopus* embryonic cDNA *X*El1a represents the mRNA for a protein with clear structural similarity to the predominant protein in mammalian liver gap junctions. The 1.5-kb transcripts that hybridize to the *X*El1a probe are not detectably represented in the pool of mRNA which is synthesized and stored during oogenesis. The putative gap junction mRNA apparently begins to accumulate at the midgastrula stage (stage 12). Its abundance increases rapidly during gastrulation and neurulation, and continues to increase at least to the tailbud stage. Therefore, these transcripts may be classified as "late" embryonic mRNAs, according to the nomenclature of Davidson (1986).

In the adult frog, nuclease protection assays that use the embryonic cDNA probe reveal closely related RNAs in the lung, the mesonephric kidney, and a variety of organs of the alimentary tract, including the liver. It should be noted that full-length cDNA clones of *Xenopus* liver gap junction mRNA have not yet been recovered. Therefore, it is not known whether the 1.5-kb liver polyadenylated RNA detected by hybridization is a product of the same gene that gives rise to the embryonic transcript. Although the cloned embryonic and liver cDNA sequences differ by one codon, no mismatch at this position is detected by S1 nuclease protection analysis of liver RNA with the embryonic probe. Consequently, it is likely that this difference represents an artifact of the cloning process or a sequence polymorphism. The near identity of the cDNA sequences and the apparent low copy number of the gene encoding them argue that the 1.5-kb transcripts in liver and embryos are the same.

Nuclease protection assays with RNA from the early tailbud stage showed that the putative gap junction mRNA has a restricted distribution in the embryo. The highest concentration of transcripts is found in the ventral portion containing ventrolateral epidermis and mesoderm and the rudiments of pharynx, heart, and endodermal structures. Within this region, the greatest abundance is found in a posterior ventral fragment that contains the liver rudiment, the ventral walls of midgut and postanal gut, and ventral mesoderm and epidermis (Nieuwkoop and Faber, 1975). Although the significance of this distribution is not known, it is possible that the gene product plays some role in morphogenesis of the respiratory, alimentary, and urogenital tract organs where it is later stably expressed. A better understanding of the embryonic distribution of the putative gap junction mRNA will require analysis by in situ hybridization.

It was surprising to find that the embryonic gap junction transcript was not represented in the maternal RNA pool. Several types of data argue for the existence of functional gap junctions in the pregastrula *Xenopus* embryo. It is clear that cell-to-cell channels exist as early as the 32-cell stage which allow diffusion of a small dye, Lucifer Yellow (Guthrie, 1984), but not of macromolecular tracers such as horseradish peroxidase and fluorescent dextrans (Hirose and Jacobson, 1979; Gimlich and Braun, 1985). Warner et al. (1984) found that microinjection of antibodies that bind to determinants on the cytoplasmic surface of mammalian gap junctions and block channel conductance (Young et al., 1987) reduced dye and ionic coupling of cells in the frog early blastula. These authors also showed that antibodies specific for the major gap junction protein of rat liver recognize *Xenopus* early embryonic proteins in immunoblot experiments. A simple prediction from the present data on RNA accumulation is that early embryonic gap junctions, while antigenically related to the liver type of junction, are the products of oocyte mRNAs which do not form stable hybrids with the *X*El1a probes. Unless such transcripts were abundant, they would be difficult to detect by low stringency hybridization on gel blots. Indeed, in more recent work (Gimlich, R. L., N. M. Kumar, and N. B. Gilula, manuscript in preparation), we have identified two maternal mRNAs that encode proteins closely related to the gap junction subunit predominant in the rat heart (Beyer et al., 1987). One of these mRNAs accumulates during oogenesis, but is absent by the neurula stage. Thus it seems that *Xenopus* embryonic gap junction proteins are the products of a family of related genes with distinct patterns of expression. The developmental significance of a switch in predominant gap junction subunit structure is a topic of current research in this laboratory.

Structural features believed to be determinants of protein folding and membrane association are well conserved among the predicted *X*El1a translation product and the known gap junction proteins of mammals (Kumar and Gilula, 1986; Paul, 1986). In particular, the number and placement of predicted transmembrane domains is similar in the frog and mammalian proteins. The amino acid sequences of these hydrophobic domains, including a putative amphipathic channel-forming helix (Milks et al., 1988), are also highly conserved. The amino terminal 220 residues show identical spacing of cysteine residues and domains rich in charged amino acids. Topological considerations based on the existence of cytoplasmic sites and four potential transmembrane helices (Unwin, 1986) predict that the gap junction protein has two extracellular domains (around residues 40–75 and 160–190 in *Xenopus*). The excellent sequence conservation in these regions is consistent with the capacity of phylogenetically diverse cells in culture to establish heterospecific gap

junctional coupling (Michalke and Loewenstein, 1971; Epstein and Gilula, 1977).

Comparison of diverse gap junction proteins also shows a consistent pattern of structural divergence. A region between the second and third putative transmembrane domains (around residues 100–140 in *Xenopus*) is highly variable in primary sequence. This domain also differs in its length between the major liver gap junction protein and that predicted for the heart gap junction in mammals (Beyer et al., 1987). The combined proteolytic and immunochemical studies of Zimmer et al. (1987) indicate that this part of the molecule is exposed on the cytoplasmic surface of the junctional membrane. The carboxy-terminal sequences (after residue 220) are highly divergent, although careful alignment reveals partial identity between the frog protein and the mammalian liver molecule. This variable region is also thought to reside on the cytoplasmic surface of the native gap junction (Zimmer et al., 1987).

Phosphorylation of gap junction proteins has been proposed to be an important modulator of junction number and conductance (Johnson et al., 1986; Saez et al., 1986). Thus, it may be significant that despite extreme sequence divergence in cytoplasmic domains, a consensus protein kinase A modification site (Krebs and Beavo, 1979) between positions 220 and 240 exists in both the human liver and frog embryonic junction proteins (at residue 227 in *Xenopus*). In contrast, the two consensus tyrosine phosphorylation sites (Patschinsky et al., 1982) in the carboxy-terminal domain of the human protein (Kumar and Gilula, 1986) are absent in the frog sequence. However, the single conserved tyrosine modification site (at amino acid 171 of the frog protein) lies in one of the putative extracellular regions. The functional significance of these primary sequence features remains to be determined.

The availability of the coding sequences for embryonic gap junction proteins will greatly facilitate detailed study of the regulation of junctional conductance and its role in development. Specific antibody probes are being developed to determine the number and distribution of different gap junction proteins in the embryo. A combination of in vitro mutagenesis and in vivo expression studies are now possible to locate the structural determinants of junctional assembly and conductance. Genomic sequences encoding diverse embryonic gap junctions have been cloned (Kumar, N. M., R. L. Gimlich, and N. B. Gilula, unpublished data) and the genetic regulatory determinants of junction biosynthesis are being identified. These approaches will form a basis for understanding the complex physiological control of cell–cell interactions during embryogenesis.

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References

Auffray, C., and F. Rougeon. 1980. Purification of mouse immunoglobulin RNAs from total myeloma tumor RNA. *Eur. J. Biochem.* 107:303–14.

Aviv, H., and P. Leder. 1972. Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. *Proc. Natl. Acad. Sci. USA.* 69:1408–1412.

Berk, A. J., and P. A. Sharp. 1977. Sizing and mapping of early adenovirus mRNAs by gel electrophoresis of S1 endonuclease digested hybrids. *Cell.* 12:721–732.

Beyer, E. C., D. L. Paul, and D. A. Goodenough. 1987. Molecular cloning of cDNA for connexin 43, a gap junction protein from rat heart. *J. Cell Biol.* 105:2621–2629.

Biggens, M. D., T. J. Gibson, and G. F. Hong. 1983. Buffer gradient gels and ^{35}S-label as an aid to rapid DNA sequence determination. *Proc. Natl. Acad. Sci. USA.* 80:3963–3965.

Blackshaw, S. E., and A. E. Warner. 1976. Low resistance junctions between mesoderm cells during development of trunk muscles. *J. Physiol.* 255: 209–230.

Brink, P. R., and M. M. Dewey. 1980. Evidence for fixed charge in the nexus. *Nature (Lond.).* 285:101–102.

Burke, J. F. 1984. High-sensitivity S1 mapping with single-stranded [^{32}P]-DNA probes synthesized from bacteriophage M13mp templates. *Gene.* 30: 63–68.

Caveney, S. 1985. The role of gap junctions in development. *Annu. Rev. Physiol.* 47:19–35.

Chase, J. W., and I. B. Dawid. 1972. Biogenesis of mitochondria during *Xenopus laevis* development. *Dev. Biol.* 27:504–518.

Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry.* 18:5294–5299.

Davidson, E. H. 1986. Gene Activity in Early Development. Academic Press, Inc., New York. 670 pp.

Dreyfuss, G., S. A. Adam, and Y. D. Choi. 1984. Physical changes in cytoplasmic messenger ribonucleoproteins in cells treated with inhibitors of mRNA transcription. *Mol. Cell. Biol.* 4:415–423.

Dumont, J. N. 1972. Oogenesis in *Xenopus laevis* (Daudin). I. Stages of oocyte development in laboratory maintained animals. *J. Morphol.* 36:153–180.

Dworkin, M. B., and I. B. Dawid. 1980. Construction of a cloned library of expressed gene sequences from *Xenopus laevis*. *Dev. Biol.* 76:435–448.

Epstein, M. L., and N. B. Gilula. 1977. A study of communication specificity between cells in culture. *J. Cell Biol.* 75:769–787.

Feinberg, A. P., and B. Vogelstein. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* 132:6–13.

Flagg-Newton, J., I. Simpson, and W. R. Loewenstein. 1979. Permeability of the cell-to-cell membrane channels in mammalian cell junctions. *Science (Wash. DC).* 205:404–407.

Fraser, S. E., C. R. Green, H. R. Bode, and N. B. Gilula. 1987. Selective disruption of gap junctional communication interferes with a patterning process in Hydra. *Science (Wash. DC).* 237:49–55.

Gilula, N. B., O. R. Reeves, and A. Steinbach. 1972. Metabolic coupling, ionic coupling, and cell contacts. *Nature (Lond.).* 235:262–265.

Gimlich, R. L., and J. Braun. 1985. Improved fluorescent compounds for tracing cell lineage. *Dev. Biol.* 109:509–514.

Glisin, V., R. Crkyenjakov, and C. Byus. 1974. Ribonucleic acid isolated by cesium chloride centrifugation. *Biochemistry.* 13:2633–2637.

Gros, D. B., B. J. Nicholson, and J.-P. Revel. 1983. Comparative analysis of the gap junction protein from rat heart and liver: is there a tissue specificity of gap junctions? *Cell.* 35:539–549.

Guthrie, S. C. 1984. Pattern of junctional communication in the early amphibian embryo. *Nature (Lond.).* 311:149–151.

Hertzberg, E. L., and R. V. Skibbens. 1984. A protein homologous to the 27,000 dalton liver gap junction protein is present in a wide variety of species and tissues. *Cell.* 39:61–69.

Hertzberg, E., D. J. Anderson, F. Friedlander, and N. B. Gilula. 1982. Comparative analysis of the major polypeptides from liver gap junctions and lens fiber junctions. *J. Cell Biol.* 92:53–59.

Hirose, G., and M. Jacobson. 1979. Clonal organization of the central nervous system of the frog. I. Clones stemming from individual blastomeres of the 16-cell and earlier stages. *Dev. Biol.* 71:191–202.

Huynh, T. V., R. A. Young, and R. W. Davis. 1984. Constructing and screening cDNA libraries in lambda gt10 and lambda gt11. *In* DNA Cloning Techniques: A Practical Approach. D. Glover, editor. IRL Press, Oxford, England. 49–78.

Ito, S., and Y. Ikematsu. 1980. Inter and intra-tissue communication during amphibian development. *Dev. Growth & Differ.* 22:247–256.

Johnson, K., P. Lampe, K. C. Hur, C. Louis, and R. Johnson. 1986. A lens intercellular junction protein, MP26, is a phosphoprotein. *J. Cell Biol.* 102: 1334–1343.

Kistler, J., D. Christie, and S. Bullivant. 1988. Homologies between gap junction proteins in lens, heart, and liver. *Nature (Lond.).* 331:721–723.

Krebs, E. E., and J. A. A. Beavo. 1979. Phosphorylation-dephosphorylation of enzymes. *Annu. Rev. Biochem.* 48:923–959.

Kumar, N. M., and N. B. Gilula. 1986. Cloning and characterization of human and rat liver cDNAs coding for a gap junction protein. *J. Cell Biol.* 103: 767–776.

Kyte, J., and R. F. Doolittle. 1980. A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* 157:105–132.

Lawrence, T. S., W. H. Beers, and N. B. Gilula. 1978. Transmission of hormonal stimulation by cell-to-cell communication. *Nature (Lond.).* 272:501–506.

Lev, Z., T. L. Thomas, A. S. Lee, R. C. Angerer, R. J. Britten, and E. H. Davidson. 1980. Developmental expression of two cloned sequences coding for rare sea urchin embryo messages. *Dev. Biol.* 76:322–340.

Loewenstein, W. R. 1981. Junctional intercellular communication: the cell-to-cell membrane channel. *Physiol. Rev.* 61:829–913.

Melton, D. A., P. A. Krieg, M. R. Rebagliati, T. Maniatis, K. Zinn, and M. R. Green. 1984. Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucleic Acids Res.* 12:7035–7056.

Messing, J. 1983. New M13 vectors for cloning. *Methods Enzymol.* 101:20–89.

Michalke, W., and W. R. Loewenstein. 1971. Communication between cells of different types. *Nature (Lond.).* 232:121–122.

Milks, L. C., N. M. Kumar, R. Houghten, N. Unwin, and N. B. Gilula. 1988. Topology of the 32 kd liver gap junction protein determined by site-directed antibody localizations. *EMBO (Eur. Mol. Biol. Organ.) J.* In press.

Mohun, T. J., S. Brennan, N. Dathan, S. Fairman, and J. B. Gurdon. 1984. Cell type-specific activation of actin genes in the early amphibian embryo. *Nature (Lond.).* 311:716–721.

Murray, S. A., and W. H. Fletcher. 1984. Hormone-induced intercellular signal transfer dissociates cyclic AMP-dependent protein kinase. *J. Cell Biol.* 98:1710–1719.

Neyton, J., and A. Trautmann. 1985. Single-channel currents of an intercellular junction. *Nature (Lond.).* 317:331–335.

Nicholson, B., R. Dermietzel, D. Teplow, O. Traub, K. Willecke, and J.-P. Revel. 1987. Two homologous protein components of hepatic gap junctions. *Nature (Lond.).* 329:732–734.

Nieuwkoop, P. D., and J. Faber. 1975. Normal Table of Xenopus laevis (Daudin). Elsevier/North-Holland, Amsterdam. 251 pp.

Patchinsky, T., T. Hunter, F. S. Esch, J. A. Cooper, and B. M. Sefton. 1982. Analysis of the sequence of amino acids surrounding sites of tyrosine phosphorylation. *Proc. Natl. Acad. Sci. USA.* 79:973–977.

Paul, D. L. 1986. Molecular cloning of cDNA for rat liver gap junction protein. *J. Cell Biol.* 103:123–134.

Pitts, J. D., and J. W. Simms. 1977. Permeability of junctions between animal cells. Intercellular transfer of nucleotides but not of macromolecules. *Exp. Cell. Res.* 104:153–163.

Saez, J., D. Spray, A. Nairn, E. Hertzberg, P. Greengard, and M. Bennett. 1986. cAMP increases junctional conductance and stimulates phosphorylation of the 27-kDa principal gap junction polypeptide. *Proc. Natl. Acad. Sci. USA.* 83:2473–2477.

Sagata, N., K. Shiokawa, and K. Yamana. 1980. A study on the steady-state population of poly (A)+ RNA during early development of Xenopus laevis. *Dev. Biol.* 77:431–448.

Sargent, T. D., M. Jamrich, and I. B. Dawid. 1986. Cell interactions and the control of gene activity during early development of *Xenopus laevis*. *Dev. Biol.* 114:238–246.

Scheller, R. H., F. D. Costantini, M. R. Kozlowski, R. J. Britten, and E. H. Davidson. 1978. Representation of cloned interspersed repetitive sequences in sea urchin RNAs. *Cell.* 15:189–203.

Schwartzmann, G., H. Wiegand, B. Rose, A. Zimmerman, D. Ben-Haim, and W. R. Loewenstein. 1981. Diameter of cell-to-cell junctional membrane channels as probed with neutral molecules. *Science (Wash. DC).* 213:551–553.

Simpson, I., B. Rose, and W. R. Loewenstein. 1977. Size limit of molecules permeating the junctional membrane channels. *Science (Wash. DC).* 195: 294–296.

Spray, D.C., and M. V. L. Bennett. 1985. Physiology and pharmacology of gap junctions. *Annu. Rev. Physiol.* 47:281–303.

Unwin, N. 1986. Is there a common design for cell membrane channels? *Nature (Lond.).* 323:12–13.

Warner, A. E. 1973. The electrical properties of the ectoderm in the amphibian embryo during induction and early development of the nervous system. *J. Physiol.* 235:267–286.

Warner, A. E., S. C. Guthrie, and N. B. Gilula. 1984. Antibodies to gap-junctional protein selectively disrupt junctional communication in the early amphibian embryo. *Nature (Lond.).* 311:127–131.

Young, J. D.-E., Z. A. Cohn, and N. B. Gilula. 1987. Functional assembly of gap junction conductance in lipid bilayers: demonstration that the major 27kD protein forms the junctional channel. *Cell.* 48:733–743.

Zimmer, P. B., C. R. Green, H. W. Evans, and N. B. Gilula. 1987. Topological analysis of the major protein in isolated intact rat liver gap junctions and gap junction-derived single membrane structures. *J. Biol. Chem.* 262:7751–7763.