Cloning and Characterization of Human and Rat Liver cDNAs Coding for a Gap Junction Protein

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Abstract. An extended synthetic oligonucleotide (58-mer) has been used to identify and characterize a human liver gap junction cDNA. The cDNA is 1,574 bases long and contains the entire coding region for a gap junction protein. In vitro translation of the RNA products of this cDNA is consistent with it coding for a 32,022-D protein. Southern blot analysis indicates that the gap junction gene is present as a single copy, and that it can be detected in a variety of organisms using the human liver cDNA as a probe. The human cDNA has been used to screen a rat liver cDNA library, and a rat liver junction cDNA clone has been isolated. The rat liver clone is 1,127 bases in length, and it has strong sequence homology to the human cDNA in the protein-coding region, but less extensive homology in the 3'-untranslated region.

Most eukaryotic tissues contain cell–cell communication pathways or electrical synapses that permit the transfer of information, in the form of small molecules, to occur between cells. Due to these junctions, all the cells in a communicating tissue potentially share a pool of small ions and metabolites, such as nucleotides, amino acids, oligosaccharides, and second messengers. However, the different types of cells in a tissue presumably retain their individuality as a result of the lack of cell–cell transfer of macromolecules, such as enzymes, DNA, RNA, etc.

This type of communication occurs through a specific type of cell surface membrane specialization, known as a gap junction (Revel and Karnovsky, 1967; Gilula et al., 1972). Gap junctions are characterized by two adjoining plasma membranes separated by a 2–3-nm space or "gap" (as viewed in thin sections) and normally exist as a polygonal lattice of 8.5-nm subunits (Makowski et al., 1977; Unwin and Zampighi, 1980).

In excitable tissues, such as the mammalian myocardium and various electrotonically coupled neurons, gap junctions provide the basis for the rapid propagation of electrical signals between cells. Gap junctional pathways, referred to as electrical synapses, provide the fundamental mechanism for synchronizing cells into a functional syncytium (Weidmann, 1952). A large body of evidence shows that gap junctional communication exists in most non-excitable tissues as well. However, little is known, although a great deal has been postulated, regarding the functional role of gap junctions in non-excitable tissues. Recently, it has been demonstrated that a selective disruption of gap junctional communication can have a substantial effect on embryonic development (Warner et al., 1984).

Gap junctions have been isolated from mammalian liver by treating plasma membrane fractions with detergents or alkali in conjunction with subcellular fractionation procedures (Goodenough and Stoeckenius, 1972; Hertzberg and Gilula, 1979; Henderson et al., 1979; Hertzberg, 1984). The junctions are isolated on the basis of their unusual insolubility. Morphologically, the isolated gap junctions are similar to those present in vivo. There is now general agreement that the isolated liver gap junction contains a single, major polypeptide with an apparent M_r of ~27,000 (Hertzberg and Gilula, 1979; Henderson et al., 1979; Finbow et al., 1980). However, other polypeptides have been reported to be present in isolated gap junctions; these include proteins of M_r 16,000 (Finbow et al., 1983) and 33,000 (Ehrhart and Chauveau, 1977).

On the basis of comparative biochemical and immunochemical studies, it is possible to conclude that some homology exists between the rat liver M_r 27,000 junction protein and junctional proteins present in other tissues and organisms (Hertzberg and Skibbens, 1984; Warner et al., 1984; Dermietzel et al., 1984; Zervos et al., 1985). However, the relationship between junction proteins from heart and liver is controversial (Gros et al., 1983; Hertzberg and Skibbens, 1984; Zervos et al., 1985; Nicholson et al., 1985). Also, it is now clear that there is no detectable molecular homology between the rat liver M_r 27,000 junction protein and the major tissue-specific junction protein (MP26) found in the eye lens fiber (Hertzberg et al., 1982; Nicholson et al., 1983).

The existence of molecular homology between junction proteins from different organisms and cell types has also been strongly indicated in some biological studies (Michalke and Loewenstein, 1971; Epstein and Gilula, 1977). When cells from different organs and different vertebrate species are paired in culture, they frequently communicate (Epstein and Gilula, 1977). This property of gap junctional expression

suggests that gap junctional polypeptides are similar in different cells. For example, when mouse myocardial cells and rat ovarian cells are co-cultured, they readily establish junctional communication pathways (Lawrence et al., 1978). Also, antibodies to the rat liver M_r 27,000 gap junction protein are effective in blocking communication in amphibian embryonic cells (Warner et al., 1984), as well as in other mammalian cell types (Hertzberg et al., 1985).

Since gap junctions are a low abundance component of the cell surface membrane, progress in the biochemical analysis of these structures has been difficult. Nonetheless, some microsequence information (for ~50 residues from the NH_2-terminus) has been obtained (Nicholson et al., 1981). This information has been useful for comparison with other potentially related proteins (Nicholson et al., 1983), and for providing an approach for identification of a gap junction cDNA. A recombinant cDNA probe for gap junction mRNAs would be most useful for identifying the gap junctional component(s) in the liver and other tissues, and for the study of junction biosynthesis, function, and regulation. The cloning and characterization of two such cDNA probes, one from human liver and one from rat liver, is described below.

Materials and Methods

Materials

All radiochemicals were purchased from Amersham Corp. (Arlington Heights, IL). Restriction enzymes were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN), Amersham Corp., or International Biotechnologies, Inc. (New Haven, CT). T4 polynucleotide kinase, nucleotides, and M13mp19 vector DNA were purchased from Pharmacia Molecular Biology Div., Pharmacia, Inc. (Piscataway, NJ). DNA polymerase I (Klenow fragment) was purchased from New England Biolabs (Beverly, MA). Chemicals were obtained either from Fisher Scientific (Fair Lawn, NJ) or from Matheson, Coleman, and Bell (Cincinnati, OH). Urea was purchased from Schwartz/Mann Biotech (Cambridge, MA).

Oligonucleotide Synthesis

DNA oligonucleotides were synthesized with either a Beckman System 1 Plus or Applied Biosystems Model 380A synthesizer. The oligonucleotides were purified by PAGE in 7 M urea (Lo et al., 1984).

Labeling of Oligonucleotide Probes

For screening cDNA libraries, the 58-mer oligonucleotide was end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase (Maniatis et al., 1982).

Screening cDNA Libraries

An adult human liver cDNA library constructed in λgt11 was kindly provided by Savio L. C. Woo, Baylor College of Medicine, Houston, TX (Kwok et al., 1985). 150,000 plaques propagated in *Escherichia coli* Y1090 were transferred to replica nylon membranes (Biotrans A; ICN Radiochemical, Irvine, CA), processed, and baked at 80°C for 2 h as described (Maniatis et al., 1982). Baked filters were prehybridized, and then hybridized to ^{32}P end-labeled 58-mer oligonucleotide probe as described (Ullrich et al., 1984). Filters were washed three times (20 min each) and autoradiographed at −70°C with Kodak XAR-5 film and a DuPont intensifying screen. One clone was identified that gave a signal on duplicate filters. This clone was plaque purified and the resulting cDNA insert subcloned into M13mp19 phage vector using standard techniques (Maniatis et al., 1982).

The cDNA selected initially by the 58-mer oligonucleotide was used subsequently to screen another aliquot of the same human liver cDNA library and, in addition, a rat liver cDNA library (Clontech, Palo Alto, CA). Approximately 150,000 plaques were screened from each library. The human liver cDNA insert was isolated from low melt agarose and oligo-labeled as described below. Hybridization with the labeled cDNA was carried out in 50% formamide, 5× SSPE, (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, pH 7.4, 1 mM EDTA), 5× Denhardt's (1× Denhardt's is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% BSA), 100 μg/ml yeast RNA per ml at 42°C for 15 h. Upon completion of hybridization, the filters were washed once (20 min) at room temperature in 1× SSC (1× SSC is 0.15 M NaCl, 0.015 M Na citrate, pH 7.0) followed by two washes at 65°C with 1× SSC. Positive clones were plaque purified, the size of the insert determined, and the phages containing the longest insert subcloned in the M13mp19 vector.

DNA Sequencing

Eco RI-digested DNA isolated from positive plaques were subcloned into M13mp19 using standard techniques (Maniatis et al., 1982). The procedure of Dale et al. (1985) was used to produce a series of overlapping deletions that were sequenced by the dideoxy chain termination method (Biggins et al., 1983). Instead of using an M13 sequencing primer, some regions were sequenced using synthetic oligonucleotides prepared against previously sequenced regions as primers for the Klenow fragment of DNA polymerase I. Both strands of the inserts were completely sequenced.

Oligo-Labeling of cDNA

Eco RI-digested phage DNA containing the human liver gap junction cDNA was separated on low-melt agarose (IBI) and the cDNA insert isolated as described (Langridge et al., 1980). The resulting cDNA was labeled with ^{32}P-dCTP to a specific activity of ~2 × 10^9 cpm/μg using random hexanucleotides as primers as described (Feinberg and Vogelstein, 1984).

RNA Isolation, Blotting, and Hybridization

RNA was isolated from biopsied human liver using a LiCl-urea procedure (Auffray and Rougeon, 1980) as modified by Mohun et al. (1984). Poly A(+) RNA was isolated using oligo dT cellulose by a batch procedure (Maniatis et al., 1982).

RNA electrophoresis was carried out using glyoxal as a denaturant as described (Thomas, 1980). RNA in the gel was transferred to Hybond N (Amersham Corp.) by capillary blotting, and baked at 80°C for 2 h.

Hybridization of ~1 × 10^6 cpm/ml ^{32}P-labeled human liver cDNA was performed as described (Thomas, 1980) at 42°C for 15 h in 50% formamide, 5× SSPE, 5× Denhardt's, 100 μg/ml yeast RNA. After hybridization, filters were washed once with 1× SSC at room temperature followed by two washes of 0.2× SSC at 65°C before exposure at −70°C to Kodak XAR-5 film with an intensifying screen. Each wash was for 20 min.

Isolation, Blotting, and Hybridization of Genomic DNA

High molecular weight DNA was isolated from human liver, rat liver, and *Xenopus* liver essentially as described (Maniatis et al., 1982). DNA from baboon, chicken, and rabbit liver was obtained from Dr. Ming-Jer Tsai, Baylor College of Medicine, Houston, TX, DNA was digested with different restriction enzymes, the fragments were separated on an agarose gel and capillary blotted to Magna Nylon 66 (Micron Seperations, Inc., NY) (Maniatis et al., 1982). After baking, the filter was prehybridized in 50% formamide, 5× SSPE, 5× Denhardt's, 100 μg/ml yeast RNA for 3 h at 42°C. Hybridization of a ^{32}P-labeled EcoRI fragment of the initially isolated human liver gap junction cDNA was carried out in the same buffer at 42°C for 15 h. After hybridization, the filter was washed with 1× SSC at room temperature, and then three times with 1× SSC at 60°C. Each wash was for 20 min.

Immunoblotting

Liver samples were homogenized in an SDS containing gel buffer, centrifuged for 5 min in a microcentrifuge and the supernatant loaded on a 12.5% SDS polyacrylamide gel as described (Warner et al., 1984). Isolated rat liver gap junctions were prepared as described (Hertzberg, 1984). Transfer to nitrocellulose paper (Schleicher & Schuell, Inc., Keene, NH) was carried out as described (Towbin et al., 1979). Blots were incubated with either affinity-purified preimmune immunoglobulin or affinity-purified immunoglobulin that recognized the gap junction protein at a 1:250 dilution essentially as described (Warner et al., 1984). The specifically bound antibodies were detected using ^{125}I-donkey anti-rabbit IgG (Amersham Corp.). Preparation and characterization of the affinity-purified gap junction antibody has been described previously (Warner et al., 1984).

In Vitro Transcription and Translation

The Eco RI fragment from the lambda phage containing the human liver

gap junction cDNA was inserted into the polylinker site of the pGEM-1 vector (Promega Biotec, Madison, WI) by standard procedures (Maniatis et al., 1982). Coding (sense) RNA was synthesized by cleaving the vector with Pvu II and performing in vitro transcription with SP6 RNA polymerase (Boehringer Mannheim, Indianapolis, IN) according to Melton et al. (1984), as modified for addition of ^{7}m GpppG to the 5′ end of RNA (Konarska et al., 1984). Similarly, antisense RNA was transcribed with T7 RNA polymerase (Bethesda Research Laboratories, Gaithersburg, MD) from Hind III–cleaved pGEM-1 vector containing the gap junction cDNA. No Hind III or Pvu II sites occur in the cDNA.

In vitro translation of the RNA was performed in a commercially available rabbit reticulocyte lysate (Bethesda Research Laboratories) according to supplier-suggested conditions using [^{35}S]methionine.

Translation was terminated by addition of SDS containing gel loading buffer. SDS gel electrophoresis of the in vitro translation products was performed in 12.5% polyacrylamide running gels with 4% polyacrylamide stacking gels as described (Dreyfuss et al., 1984). Gels were prepared for autoradiography by treatment with EN^{3}HANCE (New England Nuclear, Boston, MA) or Amplify (Amersham Corp.) using protocols suggested by the manufacturers. Gels were subsequently dried and exposed at −70°C to Kodak XAR-5 film.

Results

A human liver cDNA library was screened with a partially degenerate 58 base synthetic oligonucleotide probe (Fig. 1). The sequence of the oligonucleotide corresponded to amino acid residues 13 to 32 that were previously identified (Nicholson et al., 1981). In synthesizing the oligonucleotide, a choice of codons for the different amino acids was made according to codon usage frequencies in mammalian genes (Grantham et al., 1981). The oligonucleotide was synthesized to be complementary (antisense) to the coding (RNA) strand.

A single positive signal on duplicate filters was obtained by screening 150,000 plaques of a human liver cDNA library with the ^{32}P-labeled 58-mer. This plaque was purified to homogeneity and subcloned in both orientations into M13mp19 for sequence analysis. The nucleotide sequence, together with the deduced amino acid sequence of this cDNA is shown in Fig. 2. The cDNA was 1,574 bases long, including 16 adenylic acid residues at the 3′ end. The homology between the 58-mer oligonucleotide and the actual cDNA sequence was 81%. This indicates that the specificity of the probe was not compromised by the occasional incorrect choice of codons.

The deduced amino acid sequence appears to code for a 32,022-D protein. The assignment of a translation reading frame to the nucleotide sequence was based on both a published amino-terminal sequence of the rat liver gap junction protein (Nicholson et al., 1981) and an internal amino acid sequence that was determined in this laboratory (Zimmer, D. B., W. H. Evans, and N. B. Gilula, manuscript submitted for publication). The regions of sequence underlined in Fig. 2 indicate the actual residues that have been determined for the isolated rat liver gap junction protein.

There was perfect agreement between the deduced human protein sequence and the experimentally determined amino acid sequence of rat liver gap junctions. The amino acid composition calculated from the sequence data corresponded well to the experimentally determined values as shown in Table I. This provided further support for the deduced amino acid sequence being the actual sequence of the liver gap junction protein.

Analysis of the predicted amino acid sequence indicated that the NH_2-terminal half of the gap junction protein contained a predominance of hydrophobic amino acids, whereas the COOH-terminal half contained many charged and hydrophilic amino acids. The sequence Asn-Trp-Thr beginning at amino acid 2 was a potential glycosylation site since it matched exactly the consensus sequence, Asn-X-Thr/Ser, that has been derived for attachment of N-linked carbohydrate structures (Hubbard and Ivatt, 1981).

The cAMP-dependent protein kinases have a strong preference for serines and threonines that are located 2 residues to the COOH-terminal side of two basic amino acids (Krebs and Beavo, 1979). Two such potential cAMP-dependent phosphorylation sites were present in the gap junction sequence at positions 233 and 240.

Tyrosine phosphorylation may require the presence of a basic amino acid residue that is 7 residues on the NH_2-terminal side of the target tyrosine, together with one or more acidic residues between 1 and 6 residues away (Patschinsky et al., 1982). Several of the tyrosine residues (positions 151, 171, 243) of the human gap junction sequence exhibited this general feature.

The presence of a termination codon just upstream from the amino-terminal end of the protein indicated that a cleaved signal sequence is likely to be absent. An optimal consensus sequence for translational initiation has been described (Kozak, 1984). The GCAGGATGA found in the cDNA clone around the putative methionine initiation codon is not in good agreement with this consensus sequence. However, the cDNA does contain a purine in position −3 (3 nucleotides upstream of the initiation codon) which has been recently

Peptide VAL-ASN-ARG-HIS-SER-THR-ALA-ILE-GLY-ARG-VAL-TRP-LEU-SER-VAL-ILE-PHE-ILE-PHE-A

Codons 5′ gtn aaq vgn caq rsn acn gcn aty ggn vgn gtn tgg qtn rsn gtn aty ttq aty ttq v 3′

58 mer 3′ CTC TTG GCC GTG AGG TGT CGG TAA CCG GCC CAG ACC GAC AGA CAV TAA AAG TAR AAG T 5′

RNA 5′ GTG AAC CGG CAT TCT ACT GCC ATT GGC CGA GTA TGG CTC TCG GTC ATC TTC ATC TTC A 3′

Figure 1. Amino acid sequence of selected region of the rat liver gap junction protein and corresponding 58-mer oligonucleotide sequence. A selected region of the known amino acid sequence of the rat liver gap junction protein is shown (Nicholson et al., 1981). The possible codon choices for the different amino acids appear underneath the protein sequence. Also shown is the sequence of the 58-base-long oligonucleotide that was complementary to the coding sequence, and was used as a probe for the isolation of the human liver junction cDNA clone. The RNA sequence shown was subsequently derived from this clone. s = c,g; v = a,c; r = a,t; q = c,t; y = a,c,t; n = a,g,c,t.

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                                        30                                                  60
CCTCTGGGAAA GGG CAG CAG GAG CCA GGT GTG GCA GTG ACA GGG AGG TGT GAA TGA GGC AGG
                                        90                                                 120
ATG AAC TGG ACA GGT TTG TAC ACC TTG CTC AGT GGC GTG AAC CGG CAT TCT ACT GCC ATT
Met Asn Trp Thr Gly Leu Tyr Thr Leu Leu Ser Gly Val Asn Arg His Ser Thr Ala Ile  20
                                       150                                                 180
GGC CGA GTA TGG CTC TCG GTC ATC TTC ATC TTC AGA ATC ATG GTG CTG GTG GTG GCT GCA
Gly Arg Val Trp Leu Ser Val Ile Phe Ile Phe Arg Ile Met Val Leu Val Val Ala Ala  40
                                       210                                                 240
GAG AGT GTG TGG GGT GAT GAG AAA TCT TCC TTC ATC TGC AAC ACA CTC CAG CCT GGC TGC
Glu Ser Val Trp Gly Asp Glu Lys Ser Ser Phe Ile Cys Asn Thr Leu Gln Pro Gly Cys  60
                                       270                                                 300
AAC AGC GTT TGC TAT GAC CAA TTC TTC CCC ATC TCC CAT GTG CGG CTG TGG TCC CTG CAG
Asn Ser Val Cys Tyr Asp Gln Phe Phe Pro Ile Ser His Val Arg Leu Trp Ser Leu Gln  80
                                       330                                                 360
CTC ATC CTA GTT TCC ACC CCA GCT CTC CTC GTG GCC ATG CAC GTG GCT CAC CAG CAA CAC
Leu Ile Leu Val Ser Thr Pro Ala Leu Leu Val Ala Met His Val Ala His Gln Gln His 100
                                       390                                                 420
ATA GAG AAG AAA ATG CTA CGG CTT GAG GGC CAT GGG GAC CCC CTA CAC CTG GAG GAG GTG
Ile Glu Lys Lys Met Leu Arg Leu Glu Gly His Gly Asp Pro Leu His Leu Glu Glu Val 120
                                       450                                                 480
AAG AGG CAC AAG GTC CAC ATC TCA GGG ACA CTG TGG TGG ACC TAT GTC ATC AGC GTG GTG
Lys Arg His Lys Val His Ile Ser Gly Thr Leu Trp Trp Thr Tyr Val Ile Ser Val Val 140
                                       510                                                 540
TTC CGG CTG TTG TTT GAG GCC GTC TTC ATG TAT GTC TTT TAT CTG CTC TAC CCT GGC TAT
Phe Arg Leu Leu Phe Glu Ala Val Phe Met Tyr Val Phe Tyr Leu Leu Tyr Pro Gly Tyr 160
                                       570                                                 600
GCC ATG GTG CGG CTG GTC AAG TGC GAC GTC TAC CCC TGC CCC AAC ACA GTG GAC TGC TTC
Ala Met Val Arg Leu Val Lys Cys Asp Val Tyr Pro Cys Pro Asn Thr Val Asp Cys Phe 180
                                       630                                                 660
GTG TCC CGC CCC ACC GAG AAA ACC GTC TTC ACC GTC TTC ATG CTA GCT GCC TCT GGC ATC
Val Ser Arg Pro Thr Glu Lys Thr Val Phe Thr Val Phe Met Leu Ala Ala Ser Gly Ile 200
                                       690                                                 720
TGC ATC ATC CTC AAT GTG GCC GAG GTG GTG TAC CTC ATC ATC CGG GCC TGT GCC CGC CGA
Cys Ile Ile Leu Asn Val Ala Glu Val Val Tyr Leu Ile Ile Arg Ala Cys Ala Arg Arg 220
                                       750                                                 780
GCC CAG CGC CGC TCC AAT CCA CCT TCC CGC AAG GGC TCG GGC TTC GGC CAC CGC CTC TCA
Ala Gln Arg Arg Ser Asn Pro Pro Ser Arg Lys Gly Ser Gly Phe Gly His Arg Leu Ser 240
                                       810                                                 840
CCT GAA TAC AAG CAG AAT GAG ATC AAC AAG CTG CTG AGT GAG CAG GAT GGC TCC CTG AAA
Pro Glu Tyr Lys Gln Asn Glu Ile Asn Lys Leu Leu Ser Glu Gln Asp Gly Ser Leu Lys 260
                                       870                                                 900
GAC ATA CTG CGC CGC AGC CCT GGC ACC GGG GCT GGG CTG GCT GAA AAG AGC GAC CGC TGC
Asp Ile Leu Arg Arg Ser Pro Gly Thr Gly Ala Gly Leu Ala Glu Lys Ser Asp Arg Cys 280
                                       930                                                 960
TCG GCC TGC TGA TGC CAC ATA CCA GGC AAC CTG CCA TCC ATC CCC GAC CCT GCC CTG GGC
Ser Ala Cys ---
                                       999                                                1039
GAAGCCCTCCTCCTTCTCCCCTGCCGGTGCACAGGCCTCTGCCTGCTGGGGATTACTCGATCAAAACCTTCCTTCCCTG
                                      1078                                                1118
GCTACTTCCCTTCCTCCCGGGGCCTTCCTTTTAGGTGCTGGAGCTGGAGGGGTGGGGAGCTAGAGGCCACCTATGCCAG
                                      1157                                                1197
TGCTCAAGGTTACTGGGAGTGTGGGCTGCCCTTGTTGCCTGCACCCTTCCCTCTTCCCTCTCCCTCTCTCTGGGACCAC
                                      1236                                                1276
TGGGTACAAGAGATGGGATGCTCCGACAGCGTCTCCAATTATGAAACTAATCTTAACCCTGTGCTGTCAGATACCCTGG
                                      1315                                                1355
TTTTCTGGAGTCACAGTCAGTGAGGAGGATGTGGTAAGAGGAGGCAGAGGGCAGGGGTGCTGTGGACATGTGGGTGGAG
                                      1394                                                1434
AAGGGAGGGTGGCCAGCACTAGTAAAGGAGGAATAGTGCTTGCTGGCCACAAGGAAAAGGAGGAGGTGTCTGGGGTGAG
                                      1473                                                1513
GGAGTTAGGGAGAGAGAAGCAGGCAGATAAGTTGGAGCAGGGGTGGTCAAGGCCACCTCTGCCTCTAGTCCCCAAGGCC
                                      1552
TCTCTCTGCCTGAAATGTTACACATTAAACAGGATTTTACAGTAAAAAAAAAAAAAAAA
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Table I. Comparison of Predicted Amino Acid Composition of Gap Junction Protein with Experimental Determinants

Amino acid	Human cDNA predicted 32,000 D[a]	Rat liver 28,000 D[b]	Rat liver 28,000 D[c]
Ala	6.0	7.2	5.8
Arg	6.8	5.4	6.9
Asx	6.0	7.2	9.0
Cys	3.6	ND	ND
Glx	7.4	7.8	10.5
Gly	6.0	7.8	16.5
His	3.6	3.4	2.4
Ile	6.0	5.4	3.9
Leu	11.0	10.0	7.7
Lys	4.2	4.5	6.2
Met	2.5	3.0	1.4
Phe	4.6	6.4	3.3
Pro	4.3	4.9	3.9
Ser	8.2	9.8	9.0
Thr	4.3	5.3	4.6
Trp	2.1	ND	ND
Tyr	3.6	4.0	3.1
Val	10.3	7.8	5.8

The mole percent of each amino acid was calculated from the derived amino acid sequence. The experimental values for the gap junction protein are from (*a*) deduced protein sequence from human liver gap junction cDNA; (*b*) Henderson et al., 1979; and (*c*) Nicholson et al., 1983. ND, not determined.

shown to have a dominant effect on selection of the functional initiation codon (Kozak, 1986).

A precise fit to the polyadenylation signal, AATAAA, did not exist in the sequence of the cDNA. However, a close homologue, ATTAAA, was present 21 nucleotides upstream of the polyadenylation site; this may be responsible for directing cleavage and/or polyadenylation of the RNA.

Characterization of Gap Junction RNA

Poly A(+) RNA was isolated from human, mouse, and rat livers and subjected to blot hybridization using the 1.57-kb human liver cDNA as a probe (Fig. 3). A single major RNA species of ~1,600 bases was detected. Other minor bands of faster mobility were also detected. These may represent degradation products in the RNA sample.

The size of the RNA is consistent with the gap junction cDNA clone containing most, if not all, of the nucleotide sequence of a gap junction RNA, including the nontranslating regions.

Analysis of Genomic DNA

Total genomic DNA was isolated from the livers of human, baboon, rabbit, rat, chicken, and *Xenopus laevis*. After digestion with restriction enzymes, the fragments were examined by Southern blot analysis. As shown in Fig. 4, in the human, rabbit, baboon, and *Xenopus* DNA samples digested with Eco Rl, a single intense band of varying sizes was detected. This suggests that the gap junction gene may be present as only one copy per haploid genome.

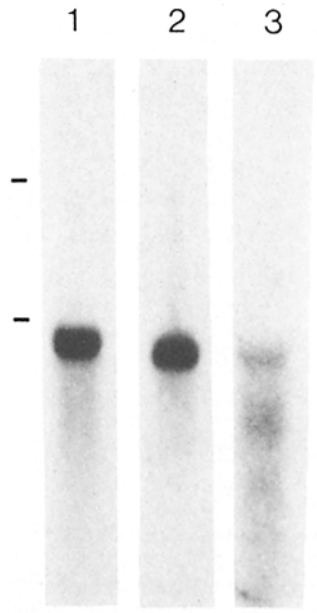

Figure 3. RNA blot analysis. 5 μg of poly A(+) RNA from human (lane *1*), rat (lane *2*), and mouse (lane *3*) livers was treated with glyoxal, separated on a 1.2% agarose gel, and transferred to Hybond N (Thomas, 1980). After prehybridization, ^{32}P-labeled human gap junction cDNA was hybridized to the blot. Conditions for hybridization were 50% formamide, 5× SSPE, 5× Denhardt's, 100 μg/ml yeast RNA at 42°C for 15 h, and the final wash was 0.2× SSC at 65°C. The size of the gap junction RNA band was estimated from RNA standards (Bethesda Research Laboratories, Gaithersburg, MD) run in adjacent lanes. Lines on the left-hand side indicate mobility of 18 S (*lower mark*) and 28 S (*upper mark*) ribosomal RNA standards.

Four fragments were detected upon hybridization of the gap junction cDNA probe to human DNA digested with Pst I. This indicated that a minimun of 3 Pst I sites exist in the gap junction gene. The restriction map of the cDNA indicated only two Pst I sites exist in the sequence. Thus, this is indicative, but not proof, that at least one intron is present in the gap junction gene, and that it contains a Pst I site.

Immunoblot Analysis of Gap Junction Protein

The molecular mass of the protein in isolated gap junctions is generally agreed to be 27,000 D as estimated by SDS polyacrylamide gels. The tissue that has been most commonly used for the isolation of gap junctions is liver from either mice or rats. The sequence of the human liver gap junction cDNA presented above suggests that the human liver gap junction protein has a calculated molecular mass of 32,022 D. One possible explanation for this discrepancy is that the human junction protein is slightly larger than the rat or mouse junction proteins. This possibility was examined by an immunoblot analysis shown in Fig. 5. Tissue homogenates were prepared from frozen rat and human liver, the proteins separated on an SDS polyacrylamide gel, transferred to nitrocellulose and probed with either gap junction-specific antibodies or preimmune antibodies. As can be observed in Fig. 5, both the human and rat contained a protein of 27,000 D that was recognized by the gap junction antibodies. This band co-migrated with the major 27,000-D protein that exists in the isolated rat liver gap junction preparation. The lower bands observed in the human liver homogenate probably reflect some degradation of the sample during its preparation. No binding was observed with the affinity-purified preimmune antibodies.

Figure 2. Sequence of the human liver gap junction cDNA. The complete nucleotide sequence of the human liver gap junction cDNA is shown. The complete protein sequence deduced from this DNA sequence is also shown and it matches with the partial protein sequence that is available (indicated by underlined regions of sequence). Arrowheads indicate the location of Pst I sites. Numbers on right-hand side refer to amino acid positions.

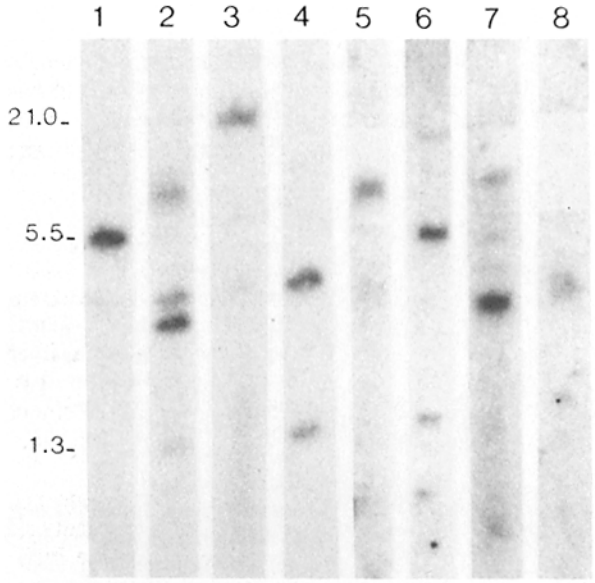

Figure 4. 10 μg of genomic DNA from the livers of various species was digested with either Eco Rl (lanes *1*, *3*, *5*, *6*, *7*, and *8*) or Pst I (lanes *2* and *4*). Fragments were separated on a 0.9% agarose gel. The filter was hybridized to a ^{32}P-labeled Eco Rl fragment of the human gap junction cDNA as described for the RNA blot (Fig. 3). Final wash was in 1× SSC at 60°C. The sizes of the DNA bands (in kb) present in the human and rabbit lanes are indicated on the left-hand side of the figure; these were determined by using Hind III–digested lambda DNA. (Lanes *1* and *2*) Human liver; (lanes *3* and *4*) rabbit liver; (lane *5*) baboon liver; (lane *6*) rat liver; (lane *7*) chicken liver; (lane *8*) *Xenopus* liver.

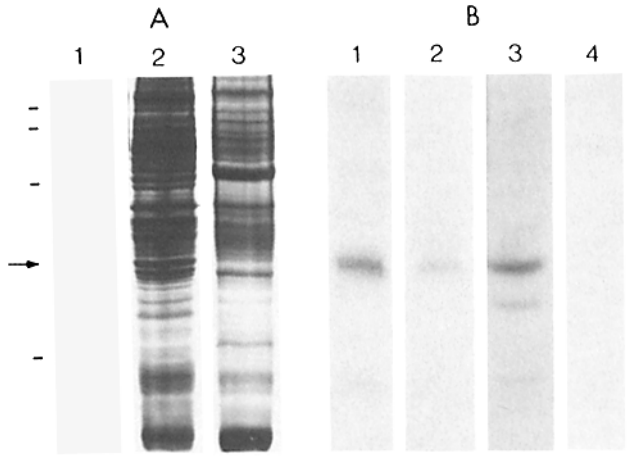

Figure 5. Immunoblot comparison of gap junction antigens from rat and human liver homogenates. Liver samples were prepared, separated, transferred to nitrocellulose, and probed with either affinity-purified gap junction antibodies (*B*, lanes *1–3*) or affinity-purified preimmune antibodies (*B*, lane *4*) as described in Materials and Methods. Specifically bound antibodies were detected using ^{125}I-donkey anti-rabbit IgG. *A* is a Coomassie Blue–stained profile of rat liver gap junctions (lane *1*), rat liver homogenate (lane *2*), and human liver homogenate (lane *3*). *B* is an identical gel probed by immunoblotting after transfer to nitrocellulose. In *B*, lane *1* contains isolated gap junctions. Lane *2* contains a rat liver homogenate. Lanes *3* and *4* contain human liver homogenates. Molecular mass standards shown on left were BSA (66 kD), catalase (57.5 kD), ovalbumin (45 kD), and trypsin inhibitor (20 kD). The arrow at the left indicates the position of the 27-kD junction protein.

In Vitro Transcription and Translation

Another possibility for the difference between the protein size determined using isolated gap junctions and the size predicted from the cDNA sequence is that some posttranslational processing may occur in vivo to produce a 27,000-D protein from a 32,022-D protein. To test this possibility, the human gap junction cDNA was subcloned into an SP6/T7 RNA polymerase vector, pGEM-1. The insert in this vector was oriented so that in vitro transcription with SP6 RNA polymerase produced a coding strand of RNA, whereas T7 RNA polymerase produced the opposite strand. The in vitro–synthesized transcripts were translated in a reticulocyte lysate system and the resulting products analyzed by SDS PAGE. Shown in Fig. 6 are the products of two different translations of in vitro–synthesized coding RNA. In one case, a major product of 32,000-D with minor bands at 26,000, 24,500, and 16,500 D, was observed. In the other case, a major product of 27,000 D was observed. This band was similar in mobility to the 27,000-D band from purified gap junctions. The same batch of reticulocyte lysate was used in all experiments. However, translations were carried out using different preparations of synthetic RNA. This experiment has been repeated a number of times and the only major products that have been detected were of 32,000 and 27,000 D. It is not clear why the two different products have been observed upon translation; however, the differences may be a reflection of the translational efficiency of the different preparations of in vitro–synthesized RNA. No translation products were observed using the noncoding strand (antisense) RNA synthesized from the T7 RNA polymerase promoter.

Comparison of Human Liver Gap Junction with Rat Gap Junction

An aliquot of the human liver cDNA library was rescreened using the original cDNA as a probe in order to confirm the DNA sequence of the human liver cDNA. A number of positive colonies were identified, one of which was purified to homogeneity and subcloned into M13mp19. The sequence of this clone was identical to the original clone, except it contained 21 adenylic acid residues at the 3′ end (compared to 16 adenylic acid residues in the original clone) and, in addition, it was one base shorter at the 5′ end. This suggested an independent origin for this clone and provided support for the sequence shown in Fig. 2.

To determine the sequence variation of the gap junction gene, a rat liver cDNA library was also screened with the original human cDNA insert. A single positive plaque was identified from screening 150,000 plaques of a rat liver cDNA library, and it was subsequently subcloned into M13mp19. The sequence of the rat liver cDNA and its comparison to the human liver cDNA is shown in Fig. 7. The rat liver gap junction cDNA was 1,127 bases long, beginning at base 401 of the human liver cDNA and ending with a 10-base-long poly (A) tail at the 3′ end.

Reverse translation of the rat liver cDNA sequence indicated excellent homology between the COOH-terminal half of the rat liver and human liver gap junction protein sequence. Only 4 amino acid changes at positions 169, 170, 171,

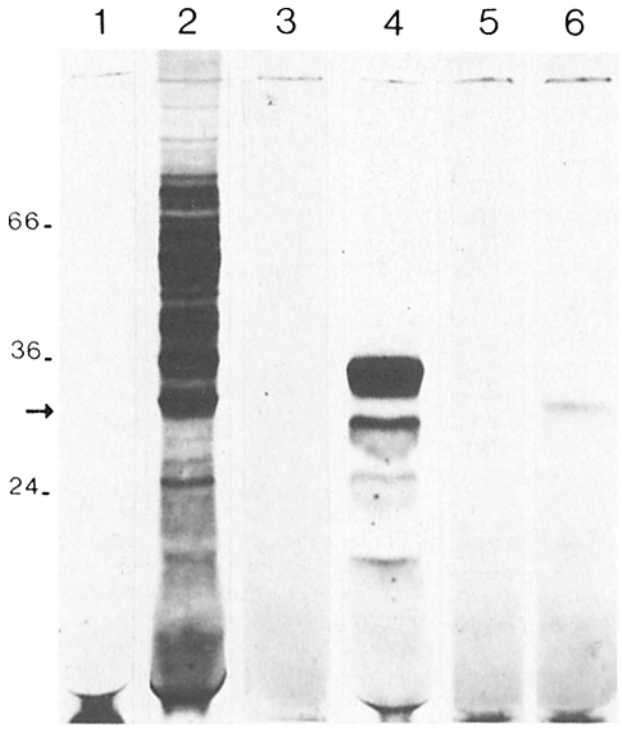

Figure 6. In vitro translation of RNA synthesized from human liver gap junction cDNA. RNA was synthesized from pGEM-1 containing the Eco R1 fragment of the human liver gap junction cDNA and translated in a reticulocyte lysate as described in Materials and Methods. Lane *1* is the translation profile observed with addition of no exogenous RNA to the translation mix. Lane *2* is the translation profile observed with rat liver poly A(+) RNA (0.5 μg/translation). Lanes *4* and *6* contain translation products of different batches of coding RNA (2 μg/translation) synthesized using SP6 RNA polymerase on Pvu II cut cDNA containing vector. Lanes *3* and *5* contain translation products of antisense RNA (2 μg/translation) synthesized using T7 RNA polymerase. On the left of the figure, molecular mass standards are indicated (in kilodaltons) and the 27,000 D position is indicated by an arrow.

and 184 (Asp, Val, Tyr, Pro in the human sequence and Glu, Ala, Phe, Ser in the rat sequence) of the human sequence appeared to be different in the two species. Apart from the change of proline to serine, these changes are conservative and unlikely to have a significant influence on gap junction structure.

At the DNA level, there was good, but not perfect, homology between the human and rat liver gap junction cDNA sequences. In the coding region of the cDNA sequence, the variation that was found was generally in the third position of a codon. This was consistent with the deduced protein sequence since such "wobbles" in the codons at the third position do not lead to variation in the protein sequence due to degeneracy in the genetic code.

In contrast, the 3′ nontranslating region had a large number of DNA sequence changes which include both additions and deletions of DNA sequence blocks, as well as base changes. Interestingly, the size of the 3′ nontranslating region in both human and rat sequences was approximately the same, even though addition and deletion of DNA sequences have occurred between the two species.

Discussion

The results presented in this study indicate that a full length cDNA coding for a human liver gap junction protein has been identified and characterized. This clone contains most, if not all, of the sequence for a gap junction RNA since there is a close similarity in the size of the gap junction RNA, as determined by Northern blot analysis, and the length of the cDNA, as determined by sequence analysis. Further proof of the full-length nature of the clone will require the isolation and characterization of genomic clones. The excellent agreement between the deduced protein sequence and the actual partial amino acid sequence derived from isolated rat liver gap junctions indicates that the cDNA does code for a gap junction protein. Consistent with this is the good agreement between amino acid composition of the isolated gap junctions and the predicted sequence.

The NH_2-terminal half of the deduced protein sequence is extremely hydrophobic, whereas the COOH-terminal half has many charged residues. This suggests that the NH_2-terminal half probably contains membrane-spanning domains, whereas the COOH-terminal portion is exposed to an aqueous environment. This is consistent with the proteolytic data obtained using isolated gap junctions (Zimmer, D. B., W. H. Evans, and N. B. Gilula, manuscript submitted for publication).

The molecular mass of the major protein present in isolated gap junctions has been estimated to be ~27,000–28,000 D (Henderson et al., 1979; Hertzberg and Gilula, 1979). The molecular mass of the gap junction deduced from the cDNA sequence is 32,022 D. The reason for this discrepancy is not known, but the following explanations can be offered: (*a*) DNA sequencing error; (*b*) differences in gap junction protein size between species; (*c*) proteolysis of gap junction protein posttranslationally or during isolation and characterization; and (*d*) anomolous migration of gap junction protein on SDS polyacrylamide gels. The first explanation is unlikely since two independently isolated clones have been sequenced, and they have identical sequences. Furthermore, a partial gap junction cDNA from a rat liver cDNA library also has almost perfect sequence homology with the human liver gap junction protein. The homology at the DNA level is almost perfect up to the end of the protein-coding sequence with variations generally occurring in the third position of a codon. However, in the 3′ nontranslating region, there is less homology with base substitution, addition and deletions, occurring in the two sequences. This is consistent with the proposed reading frame since it would be expected that the nontranslating region can vary appreciably during evolution without affecting the function of the protein.

The immunoblot analysis of rat and human liver gap junction suggests a common antigenic determinant between the two species. Furthermore, the similar molecular mass of the gap junction protein from the two species is consistent with there being considerable homology between rat and human liver gap junctions, which indicates that the second explanation is unlikely.

The most likely explanations for the discrepancy between the predicted and experimentally determined molecular masses of the gap junction protein are proteolysis or anamolous behavior of the protein on SDS polyacrylamide gels. In vitro transcription of the human cDNA insert followed by

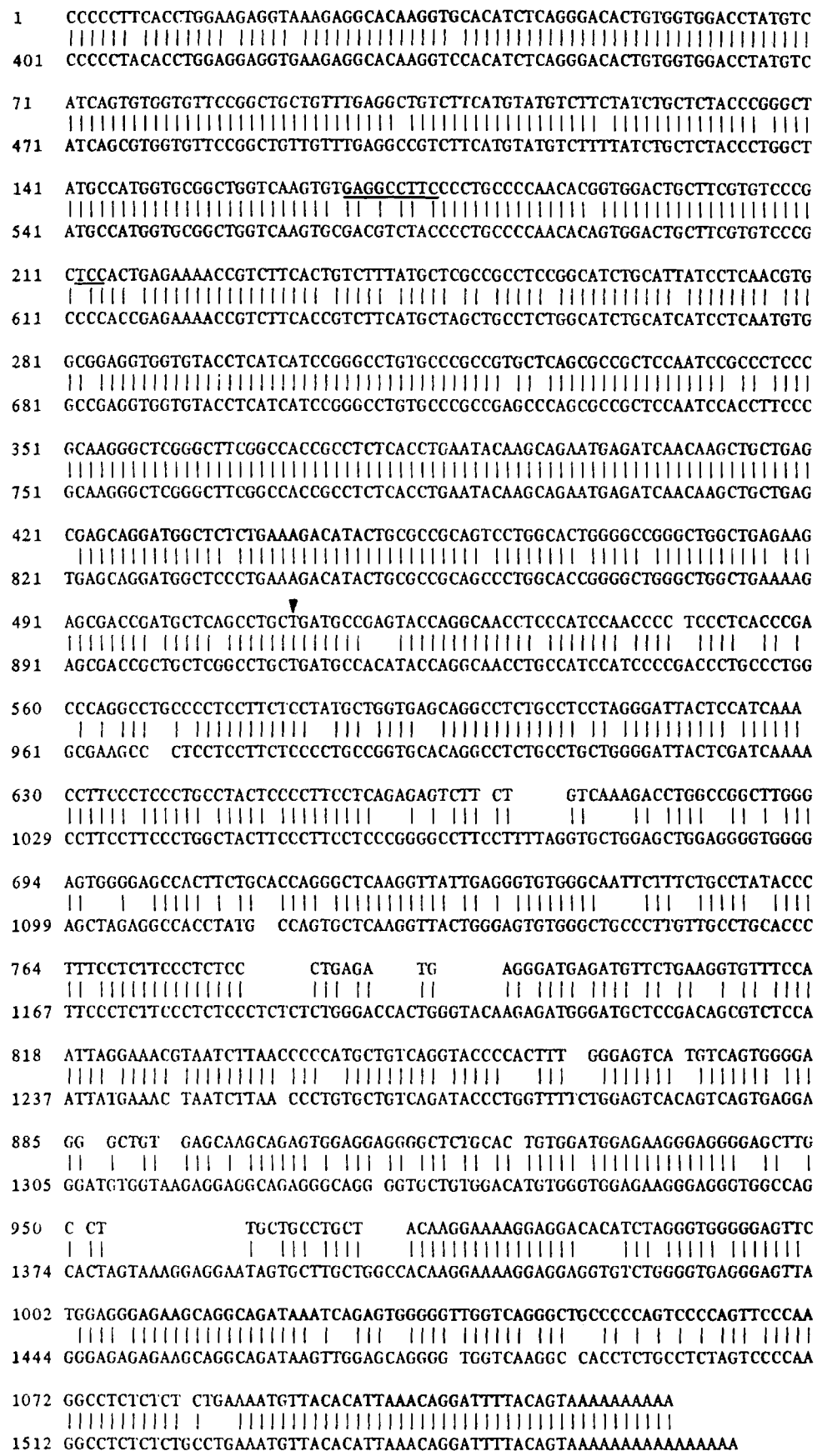

Figure 7. Comparison of rat liver gap junction cDNA sequence to gap junction human liver gap junction cDNA sequence. Shown on the uppermost line is the rat liver gap junction cDNA. Underneath is a portion of the human liver gap junction cDNA sequence (nucleotide residue 401 to 1,574) that was homologous to the rat liver gap junction cDNA sequence. Vertical bars between nucleotides indicate the presence of that nucleotide in the other cDNA sequence. The sequence has been optimally aligned using a Microgenie DNA sequence program (Beckman Instruments, Inc., Palo Alto, CA). Arrowheads indicate the predicted carboxyl terminus of the gap junctions. Regions underlined (167 to 175 and 212 to 214 of the rat liver cDNA sequence) indicate change in nucleotide sequence that result in change in protein sequence between the two species.

cell-free translation indicates that the cDNA can code for a 32,000-D protein, although occasionally, a 27,000-D protein is also observed. The reason for this variation is not known, although it is currently being studied. One possibility is that an endogenous protease in the reticulocyte lysate can, under certain conditions, digest in vitro–synthesized gap junctions. Failure to detect a 32,000-D protein in vivo could also result from a similar biologically relevant posttranslational processing event that occurs in vivo. Nonetheless, the detection of a 32,000-D translation product is consistent with the predicted size of the protein based on the cDNA sequence.

It is interesting to note that other polypeptides of different molecular masses have been proposed as components of gap junctions. Of particular relevance is a 34,000-D protein that appears to have a similar proteolytic map to the 27,000-D protein commonly reported (Finbow et al., 1980), and a 54,000-D species that has some antigenic homology to the 27,000-D protein (Warner et al., 1984; Paul, 1985).

The detection of bands upon hybridization of the human gap junction probe to genomic DNA from various species suggests that at least some portions of the gap junction gene sequence have been conserved at least down to *Xenopus* on the evolutionary scale. Such conservation would be consistent with the reported antigenic homology between rat and *Xenopus* junction proteins (Warner et al., 1984).

Gap junction conductance can be regulated by a variety of agents including H^+, Ca^{++}, and cAMP. The mechanism by which these agents act to regulate communication is not known. One attractive hypothesis, at least for cAMP, involves phosphorylation of gap junctions by a cAMP-dependent protein kinase. Addition of a subunit of a cAMP-dependent protein kinase to certain communication-defective cells in culture has been shown to increase the junctional permeability of these cells (Wiener and Loewenstein, 1983). There has been a report indicating that the junction protein can be phosphorylated in hepatocyte cultures (Saez et al., 1986). Furthermore, it has been demonstrated that a tyrosine phosphorylating protein kinase, the $pp60^{src}$ protein, can decrease junctional permeability (Atkinson et al., 1981; Azarnia and Loewenstein, 1984). Therefore, it is of interest to note that the gap junction sequence contains potential cAMP-dependent phosphorylation sites and tyrosine phosphorylation sites. These sites are localized in the COOH-terminal domain of the gap junction protein which is a region of the protein that is exposed to the cytoplasm (Zimmer, D. B., W. H. Evans, and N. B. Gilula, manuscript submitted for publication). Further experimentation will be necessary to determine whether these specific sites are phosphorylated in vivo and if they are of biological significance.

The results of this initial analysis provide conclusive evidence for the existence of a 1,574-base transcript that codes for a gap junction protein of 32,022 D. Since this coding sequence matches perfectly with the limited sequence available for the endogenous 27,000-D protein in isolated gap junctions, it is possible that the 27,000-D component could be derived from the 32,022-D component by direct proteolytic treatment of the COOH-terminal domain. However, more information will be required from genomic cloning and in vitro translation before it will be possible to understand the precise relationship between the primary transcript of the gap junction gene and the 27,000-D protein that exists in the isolated gap junctions. Thus far, preliminary RNA hybridization results have indicated that multiple transcripts with considerable size variation can be detected in RNA from different organisms (vertebrate and invertebrate) with the human liver cDNA probe. Therefore, it may be possible that the gap junction gene can produce multiple transcripts, each of which may code for a specific gap junction protein. Such diversity may account for the homologies and differences that have been reported (Warner et al., 1984; Dermietzel et al., 1984; Hertzberg and Skibbens, 1984; Paul, 1985; Nicholson et al., 1985).

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Note Added in Proof: After submission of this manuscript, a paper by D. Paul describing the cloning and characterization of a rat liver gap junction cDNA appeared in *The Journal of Cell Biology* (103:123–134, 1986). A comparison of the sequence indicates that our rat gap junction cDNA nucleotide sequence is a partial cDNA corresponding to nucleotide positions 370–148 in the sequence of D. Paul. With the exception of one nucleotide at position 1212 of our sequence (a change of a T to a C in the two sequences), the sequences are identical. This nucleotide change results in a difference in amino acid sequence (Ser in our sequence, Pro in D. Paul sequence). The significance of this change is not known. It may represent a polymorphism in the gene especially since the human gap junction cDNA also codes for a Pro at this position.

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