

Molecular Characterization and Functional Expression of the Human Cardiac Gap Junction Channel

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Abstract. Gap junctions permit the passage of ions and chemical mediators from cell to cell. To identify the molecular genetic basis for this coupling in the human heart, we have isolated clones from a human fetal cardiac cDNA library which encode the full-length human cardiac gap junction (HCGJ) mRNA. The predicted amino acid sequence is homologous to the rat cardiac gap junction protein, connexin43 (Beyer, E. D., D. Paul, and D. A. Goodenough. 1987. *J. Cell Biol.* 105:2621-2629.), differing by 9 of 382 amino acids. HCGJ mRNA is detected as early as fetal week 15 and persists in adult human cardiac samples. Genomic DNA analysis suggests the presence of two

highly homologous HCGJ loci, only one of which is functional. Stable transfection of the HCGJ cDNA into SKHepl cells, a human hepatoma line which is communication deficient, leads to the formation of functional channels. Junctional conductance in pairs of transfectants containing 10 copies of the HCGJ sequence is high (~20 nS). Single channel currents are detectable in this expression system and correspond to conductances of ~60 pS. These first measurements of the HCGJ channel are similar to the junctional conductance recorded between pairs of rat or guinea pig cardiocytes.

GAP junctions are specialized regions of adjoining cell membranes composed of numerous intercellular low resistance channels. By permitting the passage of ions and chemical mediators from cell to cell, these channels may play a major role in a wide variety of cellular processes, including embryogenesis, cellular differentiation and development, and electrotonic coupling (for review, see Bennett and Spray, 1985; Hertzberg and Johnson, 1988). In excitable tissue, gap junctions facilitate the passage of electrical activity. This electrotonic spread of current is critical to the normal function of the heart, where action potentials are propagated by current flowing through gap junction channels.

Previous studies have led to the designation of a family of proteins, known as connexins, which have been isolated from various tissues and comprise gap junction channels (Beyer et al., 1988). Members of this family are identified by their similar structural organization within the cell membrane. Complementary DNA clones which encode connexins have been isolated from several sources, including *Xenopus* embryo and liver, rat heart, liver and lens, and mouse and human liver (Beyer et al., 1987, 1988; Ebihara et al., 1989; Gimlich et al., 1988; Paul, 1986; Kumar and Gilula, 1986; Zhang and Nicholson, 1990; Gimlich et al., 1990). Within a species, the various connexins comprise a family with considerable sequence diversity, particularly in the cytoplasmic domains. Across species, however, the sequences of comparable isoforms are extremely well conserved. For example, amino acid sequences of rat con-

nexin32, connexin43, and connexin46 proteins are only 56% identical in the most conserved region, while rat and human connexin32 proteins are 98% identical over their entire length (Beyer et al., 1988; Paul, 1986; Kumar and Gilula, 1986). This generation of diversity between tissues, but conservation of protein sequence across species, suggests that functional attributes of specific gap junction channels are tissue specific.

Because of the important role played by the cardiac gap junction in maintaining the normal electrophysiology of the heart, and the possibility of alterations in cellular coupling contributing to arrhythmogenesis (Ikeda et al., 1980; Kleber et al., 1986, 1987; Spach et al., 1982), we have been interested in characterizing the human cardiac gap junction (HCGJ)¹ channel. In this report, we show that a single gene encodes the HCGJ protein. A processed pseudogene is also present in the genome. The HCGJ gene gives rise to a single 3.1-kb mRNA transcript. The predicted amino acid sequence is 97% identical to the rat cardiac gap junction protein, connexin43. HCGJ mRNA is detectable in early fetal hearts as well as in adult cardiac tissue. Transfection of the cDNA encoding the HCGJ protein into SKHepl cells, a human hepatoma cell line which is communication deficient (Eghbali et al., 1990), results in clones that are extremely well coupled, as evaluated by both the intercellular diffusion of Lucifer Yel-

1. *Abbreviations used in this paper:* HCGJ, human cardiac gap junction; PCR, polymerase chain reaction; PKA, protein kinase A.

low and direct electrophysiological measurements. Single channel recordings reveal that the unitary conductance of channels formed by the HCGJ protein is similar to that recorded between pairs of neonatal rat and adult guinea pig heart cells (Burt and Spray, 1988a; Rook et al., 1988; Rudisuli and Weingart, 1989). These unitary conductances are markedly lower than those expressed in the same cell type transfected with rat connexin32 cDNA (Eghbali et al., 1990), indicating that connexin type, rather than cellular environment, is a major determinant of channel size.

Materials and Methods

Tissue Sources and Isolation of RNA

Human fetal cardiac tissue was obtained from elective abortions and was generously provided by S. Kohtz (Mt. Sinai School of Medicine, New York) and P. Allen (Brigham and Women's Hospital, Boston, MA). Normal human adult samples were obtained from potential cardiac transplantation donors, kindly provided by E. Horn (Columbia-Presbyterian Medical Center, New York). Pathologic adult human cardiac samples were from explanted hearts of cardiac transplantation recipients, and were provided by M. Thompson (University of Pittsburgh School of Medicine, Pittsburgh, PA). Total cellular RNA was isolated as described (Chomczynsky and Sacchi, 1987).

Isolation of cDNA Clones

A cDNA library was constructed in lambda gt10 using 5 µg poly(A)⁺ RNA obtained from a 15-wk human fetal left ventricle. The library was initially screened with a radio labeled cRNA probe (nucleotides 226–265) derived from the rat cardiac gap junction cDNA, G2A (Beyer et al., 1987). Approximately 5 × 10⁵ plaques lifted onto Gene-Screen filters (New England Nuclear, Boston, MA) were initially screened and a single positive clone was identified, (designated HCGJ7). Filters were prehybridized in 50% formamide, 6× SSC (1× SSC is 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0), 0.1% SDS, 1 mM EDTA, 5× Denhardt's solution, with 100 µg/ml yeast tRNA, for 4 h at 50°C, and hybridized overnight in the same buffer after the addition of probe at a concentration of 2 × 10⁵ cpm/ml. Filters were washed in 1× SSC, 0.1% SDS for 1 h at 50°C. Because this insert did not contain a full-length cDNA, the library was rescreened with a more 3' portion of G2A, corresponding to nucleotides 1,114–1,370. This fragment was radio labeled utilizing random hexanucleotides and the Klenow fragment of DNA polymerase (Pharmacia Fine Chemicals, Piscataway, NJ). For the cDNA probe, filters were prehybridized at 65°C in 5× SSC, 50 mM sodium phosphate pH 7.4, 1× Denhardt's solution, with 100 µg/ml denatured salmon sperm DNA, followed by hybridization in the same solution with the addition of 2 × 10⁵ cpm/ml of probe, and washed in 2× SSC, 0.2% SDS at 65°C for a total of 1 h. Two additional positive clones were identified (designated HCGJ16 and HCGJ8).

Polymerase Chain Reactions (PCRs)

Anchored PCR was performed by a modification of the procedure described by Frohman et al. (1988). First strand cDNA was prepared using 1 µg of total RNA prepared from an explanted human left ventricle. The RNA was heated to 65°C for 10 min, and then rapidly cooled on ice. The volume was brought up to 16 µl, containing 50 mM Tris-HCl, pH 8.3, 150 mM KCl, 10 mM MgCl₂, 5 mM DTT, 0.5 mM (each) dNTPs, 15 U of AMV reverse transcriptase (Life Science Associates, Bayport, NY), and 300 pmol of the primer 5'-CGCGGAATTCCTCCGGGCGCGC(T)₁₂-3', and incubated at 42°C for 1 h. After first strand synthesis, the entire reaction was diluted to 100 µl containing the standard PCR reaction mix of 100 pmol of each primer, 50 mM KCl, 10 mM Tris-HCl, pH 8.5, 1.5 mM MgCl₂, 0.1% gelatin, 200 µM (each) dNTPs, and 2.5 U of Taq polymerase (Cetus Corp., Emeryville, CA). The 5' specific oligonucleotide primer corresponded to nucleotides 2,504–2,525 of the cDNA, and the 3' primer was the anchor portion of the reverse transcription primer, 5'-CGCGGAATTCCTCCGGGCGCGC-3'. The sample was subjected to 30 cycles of PCR, denaturing at 94°C for 30 s, annealing at 54°C for 30 s, and extending at 72°C for 1 min. A final 10-min extension period was then added. The major PCR

product was isolated from an agarose gel, purified, and cloned into the vector PTZ19R (Pharmacia Fine Chemicals).

For amplification of genomic DNA, 5 µg of total human genomic DNA was denatured at 95°C for 5 min and then rapidly cooled on ice. Reactions were then carried out in a final volume of 100 µl which contained the standard PCR reaction mix. These samples were cycled as described above, except that denaturing was for 1 min, and annealing was at 62°C. Primers corresponded to nucleotides 149–173 (sense) and 507–524 (antisense) of HCGJ16.

DNA Sequence Analysis

Phage DNA from positive clones was purified by the plate lysate method (Maniatis, 1982), and the Eco RI inserts were subcloned into the plasmid vector Bluescript KS⁺ (Stratagene Cloning Systems, La Jolla, CA). Inserts were sequenced by a combination of nested deletions, using the Erase-A-Base system (ProMega Biotec, Madison, WI), as well as by utilizing custom-designed oligonucleotide primers for dideoxy chain termination reactions, synthesized in the Albert Einstein College of Medicine Shared DNA Synthesis Facility. Because of the overlapping nature of nested deletions, each nucleotide was sequenced at least two times. Sequence data were analyzed using Staden computer software (Pearson and Lipman, 1988).

Northern Blots

Total RNA samples (10 µg) were electrophoresed on 0.7% agarose/formaldehyde gels and capillary blotted onto Nytran membranes (Schleicher & Schuell, Keene, NH). Membranes were prehybridized at 55°C in 5× SSC, 50 mM sodium phosphate pH 7.4, 1× Denhardt's solution, 2% SDS with 100 µg/ml denatured salmon sperm DNA, then hybridized in the same solution with the addition of 1 × 10⁶ cpm/ml radiolabeled oligonucleotide probe. Membranes were washed with 2× SSC, 2% SDS for 30 min at room temperature, followed by a 5-min wash at 55°C. The radiolabeled oligonucleotide probe corresponded to the complement of nucleotides 1,280–1,300 of the HCGJ cDNA, and was prepared with γ-³²P-ATP and polynucleotide kinase according to the manufacturer's directions (Pharmacia Fine Chemicals).

Southern Blots

Total human genomic DNA (15 µg) was digested to completion with either Eco RI, Hind III, or Xba I and electrophoresed on 1% agarose-Tris acetate-EDTA (TAE) gels and capillary blotted onto Nytran membranes (Schleicher and Schuell). Filters were prehybridized at 65°C in 5× SSC, 50 mM sodium phosphate, pH 7.4, 1× Denhardt's solution, with 100 µg/ml denatured salmon sperm DNA, followed by hybridization in the same solution with the addition of 2 × 10⁵ cpm/ml of probe. Filters were washed in 2× SSC, 0.2% SDS at 65°C for a total of 1 h. For analysis of transfectants, genomic DNA was prepared from confluent 100-mm tissue culture plates (Gross-Bellard et al., 1972) and digested to completion with Bam HI.

Western Blots

Tissue culture cells were harvested by scraping confluent 100-mm plates and pelleting in a microfuge. Pellets were washed with ice-cold PBS, resuspended in a small volume of water and sonicated, then solubilized by boiling in Laemmli sample buffer (Laemmli, 1970). Homogenates from rat myocardium and liver were similarly prepared. 37 µg of protein was loaded per lane, as determined by protein assay (Bio-Rad Laboratories, Richmond, CA). An immunoblot was made using a polyclonal antibody directed against a synthetic polypeptide corresponding to residues 346–363 of the rat connexin43 protein (Yamamoto et al., 1990), followed by ¹²⁵I-labeled protein A.

Cell Culture and DNA Transfections

SKHepl cells were maintained in DME supplemented with 10% FBS (HyClone Laboratories, Logan, UT), 50 IU/ml penicillin, 50 µg/ml streptomycin, and 2 mM L-glutamine. Cells were transfected with the expression vector pGF1 and the dominant selectable marker pSV7Neo (Colby and Shenk, 1982). pGF1 is derived from the Rous sarcoma virus expression vector, pRSVcat (Gorman et al., 1982). The parental vector was digested at its unique Hind III site, filled in, and religated to create a new Nhe I site. This construct was then digested with both Nhe I and Bam HI to remove the CAT sequence, and the large fragment was isolated. A second fragment contain-

ing nucleotides 1-1,817 of the HCGJ cDNA, which includes the 5' untranslated region, the entire coding region, and 510 nucleotides from the 3' untranslated region, was isolated as an Xba I-Kpn I fragment. The internal Xba I site does not cleave due to overlapping dam methylation. A third fragment, containing the SV40 early region splice and polyadenylation signals from pSV2Neo (Southern and Berg, 1982) was isolated as a Kpn I-Bam HI fragment. These three fragments were gel purified, and ligated to create pGF1 (see Fig. 3 a). Cells were transfected with 25 μ g pGF1 and 5 μ g pSV7Neo, using standard calcium phosphate coprecipitation techniques (Graham and van der Eb, 1973). Selection was begun 24 h after transfection with the addition of G418 (Geneticin; Gibco Laboratories, Grand Island, NY) at 400 μ g/ml to the media, and individual colonies were subsequently analyzed.

Electrophysiological Analysis of HCGJ Transfectants

Clones were screened for the presence of gap junction channels by the ability to transfer dye. Individual cells were injected with Lucifer Yellow (5% wt/vol in 150 mM LiCl), using overcompensation of the negative capacitance circuit on a W-P Instruments electrometer. Fields were visualized under both phase contrast and fluorescent illumination using an FITC filter combination. Cells were photographed on Kodak TMAX 400 film using a constant exposure time of 30 s, beginning 1 min after injection.

To assess electrical coupling, cell pairs were obtained by freshly dissociating pure populations of confluent cultures onto 1-cm diameter glass coverslips. Coverslips were transferred to the stage of a Nikon Diaphot microscope at 3 h to 3 d after splitting, where experiments were performed at room temperature while continuously exchanging the bath solution (133 mM NaCl, 3.6 mM KCl, 1.0 mM CaCl₂, 0.3 mM MgCl₂, 16 mM glucose, 3.0 mM Hepes, pH 7.2). Each cell of a pair was voltage clamped using heat-polished patch type pipettes filled with a solution at pCa 8 (135 mM CsCl, 0.5 mM CaCl₂, 2 mM MgCl₂, 5.5 mM EGTA, 5 mM Hepes-KOH, pH 7.2). Pipette resistance was generally 2-5 MOhms. High resistance seals (>10⁹ Ohms) were formed on each cell with the aid of gentle suction and access to the cell interior was then gained by brief strong suction applied to the patch pipette. Series resistance compensation, which had negligible effects on recordings, was rarely employed. Cells were voltage clamped at holding potentials of -40 mV and command steps were presented alternately to each cell of the pair. Junctional current (I_j) was measured as the current evoked in one cell by the voltage step in the other (V_j). Junctional conductance (g_j) = I_j/V_j . Cell pairs were uncoupled by the addition of halothane (1.5-2.0 mM) to the bathing solution, as described by Burt and Spray (1989). Under conditions of reduced g_j , single channel currents could be recorded at high gain by imposing a constant transjunctional driving force (20-50 mV). Currents and voltages were continuously monitored on a four channel chart recorder (Gould Instruments, Cleveland, OH) and recorded on video tape after digitization (Neurocorder Corp., Neurodata Inc., New York).

Results

Isolation and Sequence Analysis of HCGJ cDNA Clones

Using cRNA and cDNA probes derived from the rat connexin43 clone G2A (see Materials and Methods), three independent clones were isolated from the human cardiac cDNA library, and sequenced in their entirety. The first two clones, designated HCGJ7 and HCGJ8, overlapped with a single base pair discrepancy. Compared to both the rat connexin43 sequence, as well as HCGJ8, the HCGJ7 clone contained a single base deletion which disrupted the reading frame, and is presumed to be a cloning artifact. The 3' end of HCGJ8 contained an extraneous fragment of 806 nucleotides appended onto it, which did not hybridize to cardiac RNA. To independently demonstrate the true 3' end of the HCGJ transcript, we utilized the anchored PCR procedure (see Materials and Methods). Using a 5' specific primer corresponding to nucleotides 2,504-2,525 of the consensus cDNA, along with the 3' non-specific primer, a prominent

band of 600 bp was generated. This product was isolated and sequenced in its entirety, and is identical to HCGJ8 from nucleotides 2,504 through 3,038, followed by a poly(A) tail of 31 residues, beginning 21 bp downstream from the polyadenylation signal (see Fig. 2). Finally, a second region of unexpected sequence was found in the 5' end of clone HCGJ16, which differed from the consensus cDNA for its first 524 nucleotides, and then was identical to the consensus throughout its remaining 1,042 nucleotides. Several lines of evidence suggest that this portion of HCGJ16 is derived from the first intron of the gene, and represents cloning of an unprocessed transcript. Examination of the sequence of HCGJ16 at the point of divergence from the consensus cDNA reveals a perfect splice junction acceptor site (Mount, 1982). Furthermore, the location of this putative intron-exon border is identical to that reported for the rat liver gap junction gene, connexin32 (Miller et al., 1988), located in the 5' untranslated region, 16 nucleotides upstream from the initiation codon. A schematic of these sequences is shown in Fig. 1. Genomic hybridizations, described below, demonstrate that the same bands are recognized by probes which reside close to either side of the proposed splice junction. In addition, PCR using oligonucleotide primers derived from opposite sides of the splice junction (nucleotides 149-173 and 507-524 of HCGJ16) generates the correct 375-bp product when using genomic DNA as a template (data not shown).

The HCGJ nucleotide and predicted amino acid sequences are shown in Fig. 2. The composite cDNA contains 3,069 nucleotides, and in contrast to the rat connexin43 sequence (Beyer et al., 1987), includes the entire 3' untranslated region and poly(A) tail. The size of HCGJ mRNA thus appears to be similar to that of rat connexin43 mRNA, an estimate which was based upon electrophoretic mobility (Beyer et al., 1987). There is an open reading frame of 1,146 bp beginning at nucleotide 158, followed by a 3' untranslated region of 1,734 bp plus the poly(A) tail. The open reading frame encodes a protein of 382 amino acids with a predicted molecular weight of 43,009 D. According to proposed terminology, the HCGJ protein would be named human connexin43. Compared to the rat connexin43 sequence, there is 84% homology at the nucleotide level, beginning in the 5' untranslated region, and continuing several hundred bp into the 3' untranslated region. Interestingly, after this point, no significant homology to the rat connexin43 cDNA is found. At the amino acid level, there is 97% homology. Of the 382 amino acids, there are nine differences between the two species, as indicated in Fig. 2.

A number of structural domains and potential modification sites for the connexin family of proteins have been predicted based upon primary sequence analyses. A hydrophobicity plot of the human cardiac gap junction sequence is similar to that found for rat connexin43, consisting of four hydrophobic membrane spanning domains which are separated by hydrophilic segments (Beyer et al., 1987). The two hydrophilic extracellular loops (encompassing amino acids 44-68 and 185-207 of connexin43) are highly conserved among all connexin isoforms. The human cardiac protein preserves the precise spacing of the three cysteine residues found within each loop. These particular amino acids may form disulfide bonds with cysteines in adjacent hemichannels, contributing to the formation of the intercellular junc-

Human clone HCGJ7 (nucleotides 125-160) TACTTTTAAAGCAAAGAGTGGTGCCAGGCAACATG
 Human clone HCGJ16 (nucleotides 507-543) tctctttgtttctttcagGTGGTGCCAGGCAACATG
 Rat connexin32 gene ctgcctcttgtttccagGTGTGAATGAGGCAGGATG
 Consensus splice $\binom{t}{c}_{n>5} n^{c}_{t} agG$

Figure 1. HCGJ acceptor splice site. Alignment of HCGJ clones HCGJ7 (nucleotides 125-160) and HCGJ16 (nucleotides 507-543), isolated from a human fetal cardiac cDNA library, shown along with the rat connexin32 gene splice site and the consensus acceptor splice site sequence. Nucleotides which are known or proposed to be from intervening sequence are shown in lower case. The initiation codons are shown in boldface. The portion of HCGJ16 that differs from HCGJ7 ends in a consensus splice site, suggesting it is derived from intervening sequence. See text for details.

tion. Interestingly, neither the human nor rat connexin43 amino acid sequences exhibit strong consensus sites for modification by either cAMP-dependent protein kinase (PKA) or cGMP-dependent protein kinase. Although the amino acids flanking serine-257 in the rat connexin43 sequence (Pro-Ser-Lys) are favorable for cGMP-dependent phosphorylation, the two neighboring basic residues found in the consensus

sequence are absent (Edelman et al., 1987). Furthermore, serine-257 in the rat protein is replaced by alanine in the human sequence, disrupting the site entirely. Both the human and rat connexin43 sequences contain numerous potential protein kinase C modification sites (Woodgett et al., 1986) located within the carboxy-terminal cytoplasmic domain (residues 364, 368, and 372), and therefore available for intra-

CGGTGAGAAAGTACCAACAGCAGCGGAGTTTTAAACTTTAAATAGACAGGCTTGAGTGCCTGAACCTGGCTTTTCATTTTACTTCATCCTCCAAGGAGTTCATCACTTGGCGTGACT 120
 1 M G D W S A L G K L L D K V Q A Y S T A G G K V W L S V
 TCACTACTTTTAAAGCAAAGAGTGGTGCCAGGCAACATGGGTGACTGGAGCGCCTTAGGCAAACCTCTGACAAGGTTCAAGCCTACTCAACTGCTGGAGGGAAGTGTGGCTGTCAGT 240
 29 L F I F R I L L L G T A V E S A W G D E Q S A F R C N T Q Q P G C E N V C Y D K
 ACTTTTCATTTCCGAATCCTGCTGCTGGGGACAGCGGTTGAGTCAGCCTGGGAGATGAGCAGTCTGCCTTTCGTTGTACACTCAGCAACCTGGTTGTGAAAATGCTGCTATGACAA 360
 69 S F P I S H V R F W V L Q I I F V S V P T L L Y L A H V F Y V M R K E E K L N K
 GTCCTTCCCAATCTCTCATGTGCGCTCTGGGTCTGCAGATCATATTGTGTCTGTACCCACACTCTGTACCTGGCTCATGTGTTCTATGTGATGCGAAAGGAAGAACTGAACAA 480
 109 K E E E L K V A Q T D G V N V D M H L K Q I E I K K F K Y G I E E H G K V K M R
 GAAAGAGGAAGAACTCAAGGTTGCCCAAACCTGATGGTGTCAATGTGGACATGCACCTTGAAGCAGATTGAGATAAAGAAGTCAAGTACGGTATTGAAGAGCATGGTAAAGTGAAGTCCG 600
 149 G G L L R T Y I I S I L F K S I F E V A F L L I Q W Y I Y G F S L S A V Y T C K
 AGGGGGTTGCTGGCAACCTACATCATCAGTATCCTCTCAAGTCTATCTTTGAGGTGGCCTTCTTGTGATGCCAGTGGTACATCTATGGATTACGCTTGAGTGTCTTACACTTGCAA 720
 189 R D P C P H Q V D C F L S R P T E K T I F I I F M L V V S L V S L A L N I I E L
 AAGAGATCCCTGCCACATCAGGTGGACTGTTTCCTCTCTCGCCCCACGGAGAAAACCATCTTCATCATCTTATGCTGGTGGTGTCTTGGTGTCCCTGGCCTTGAATATCATTGAACT 840
 229 F Y V F F K G V K D R V K G K S D P Y H A T S G A L S P A K D C G S Q K Y A Y F
 CTTCTATGTTTTCTTCAAGGGCGTTAAGGATCGGGTTAAGGAAAGAGCGCACCTTACCATCGCACCAGTGGTGGCTGAGCCCTGCCAAGACTGTGGGTCTCAAAAATATGCTTATTT 960
 269 N G C S S P T A P L S P M S P P G Y K L V T G D R N N S S C R N Y N K Q A S E Q
 CAATGGCTGCTCTCACCACCGCTCCCTCTCGCCTATGTCTCCTCTGGTACAGCTGGTACTGGCGACAGAAACAATCTCTTCTGCGCAATTACAACAGCAAGCAAGTGAGCA 1080
 309 N W A N Y S A E Q N R M G Q A G S T I S N S H A Q P F D F P D D N Q A N S K K L A
 AAAGTGGCTAATTACAGTGCAGAAACAACTGAATGGGGCAGCGGGAACCCATCTCTAACTCCCATGACAGCCTTTTGATTTCCCGGATGATAACCAGAAATCTTAAAAACTAGC 1200
 349 A G H E L Q P L A I V D Q R P S S R A S S R A S S R P R P D D L E I *
 TGCTGGACATGAATTACAGCCACTAGCCATTGTGGACCAGCAGCCTTCAAGCAGAGCCAGCTGCGCCAGCAGCAGACCTCGGCCGATGACCTGGAGATCTAGATACAGGCTTGAAA 1320
 GCATCAAGATTCCTCAATTTGGGAGAAGAAAAGTGGCTGAGAAAGTGCACACAGGTTGTAATTTGATCCGGTGGAGGTGCTACTCAACAGCCTTATTCATGAGGCTTAGAAAACA 1440
 CAAAGACATTAGAATACCTAGGTTACCTGGGGTGTATGGGGTAGATGGGTGGAGGGGGGATAAGAGAGGTGCATGTTGGTATTTAAAGTAGTGGATTCAAAGAAGTATAGATATA 1560
 AATAAGAGTCCATTAGGTGATACATAGATAAGGGCTTTTCTCCCGCAAACACCCCTAAGAATGGTCTGTGATGAAAGAGCGGGTGAATTTGGCTAAAATTTTTGTTTTA 1680
 CCAAGAACTGAAATAATCTGGCCAGGAATAAATCTCCTGAACATCTTAGCTTTTCAACAAGAAAAGACAGAGGATGTCCTTAAAGTCCCTGCTAAAACATTCCTATGTTAAA 1800
 TTTGCACCTTGAAGGTAAGCTTTCTAGGCTGACCTCCAGGTGCTCAATGGACTTTGGCTACTATATTTTTTATCTTGGTATCAGTTAAAATTCAGACAGGCCACAGAAATAAGAT 1920
 TTTCCATGCATTGCAAAATACGTATATTTCTTTTCCATCCACTTGCACAATATCATTACCATCACTTTTTCATCATCTCCAGCTACTACTACATTCATTTAATGGTTTCTGTAACAT 2040
 TTTAAGACAGTGGGATGCTACTTAACATTTTTTTTTTTGAGCTAAAGTCAAGGAAATCAAGCCATGCTTAATTTAACAATCACTTATATGTTGTGCGAAGAGTTGTTTGTGTTT 2160
 CATGATTTGGTACAAGCAGATACAGTATAAACTCACAACACAGATTTGAAAATAATGCACATATGGTGTCAAATTTGAACCTTCTCATGGATTTTGTGGTGGGGCCAAATGGTG 2280
 TTTACATTAATAATCTTCCTGCTGTCGCAAGTAAAGCACACTTTTTTTTCTCCTAAAATGTTTTTCCCTGTATCTTATATGATAGTGGTTTGTGTAATATGATCTTTATTTTCT 2400
 CTCCTTTTTTAGGATATAGCAGTAATGCTATTACTGAAATGAATTTCCCTTTTCTGAAATGTAATCATTGATGCTTGAATGATAGAATTTTAGTACTGTAACAGGCTTTAGTCATTAA 2520
 TGTGAGAGACTAGAAAAATGCTTAGAGTGGACTATAAATGTGCTAAAATGAATTTTGCAGTAACCTGGTATCTTGGGTTTTCTACTTAATACACAGTAATTCAGAACTGTATTCT 2640
 ATATGAGTTAGCAGTCTTTGGAGTGACCAGCACTTTGATGTTGCACTAAGATTTTTATTGGAATGCAAGAGGTTGAAAGAGGATTCAGTAGTACACATACTAATTTATTT 2760
 GAACTATATGTTGAAGACTTCTCCAAATGCCTTTTTTAAACTCATCACAAGATTTGGTGAATAATGCTGAGTATGACACTTTCTTCTTCATGATGACACTACAT 2880
 AACAGTTTTGTACAAATGAAAATTAATTTGTTGACATTCATGTTAAACTACGGTCTGTTTCAAGCTTCAATGATGTAATGTAGACCTAGTCCATCAGATCATGTGTTCTGGAGAGT 3000
 GTCTTTATTCATAAAGTTTTAATTTAGTATAAACATAAAAAAATAAAAAAATAAAAAAATAAAAAAATAAAAAAATAAAAAAATAAAAAAATAAAAAAATAAAAAAATAAAAAA 3069

Figure 2. HCGJ cDNA and predicted amino acid sequences. The initiation codon, termination codon, and polyadenylation signal are underlined. Amino acids that differ from the rat connexin43 sequence are also underlined. Nucleotides are numbered on the right side and amino acids are numbered on the left side. These sequence data are available from EMBL/GenBank/DBJ under accession number X52947.

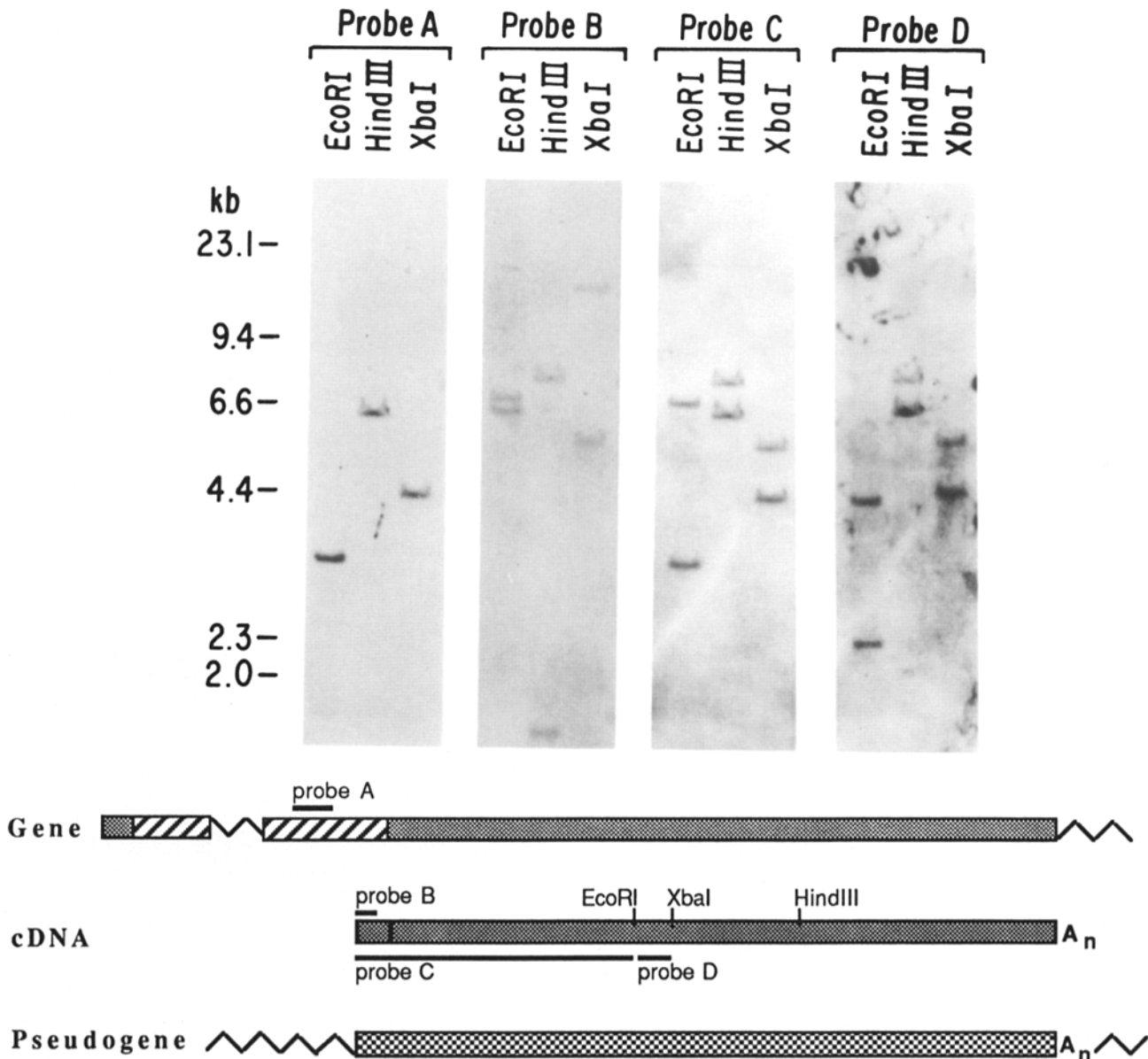


Figure 3. Genomic hybridizations and deduced gene organization. (*top*) A Southern blot was prepared from human genomic DNA and hybridized sequentially with probes A, B, C, and D under high stringency conditions (see Materials and Methods). Molecular weight markers are indicated on the left. (*bottom*) The deduced partial genomic organization is shown schematically, along with the location of each probe. Probe A is derived from intervening sequence and is gene specific, whereas probes B, C, and D, derived from the cDNA, recognize both the gene and pseudogene. Probe C recognizes the same bands as the adjacent probe A as well as those contained within the pseudogene. Although Probe C contains Probe B, it recognizes bands containing the first exon poorly, likely due to its limited overlap. ▨, sequence from exons; ⚡, intervening sequence; ▩, the pseudogene.

cellular regulation. Both species also exhibit a potential tyrosine phosphorylation site in the cytoplasmic tail, at amino acid 265 (Patchinsky et al., 1982).

Southern Blot Analysis

To estimate the number of genes that encode the HCGJ protein, four different cDNA probes were sequentially hybridized under high stringency conditions with human DNA digested with three restriction endonucleases. The location of these probes, and the genomic organization deduced from these hybridizations, are shown in Fig. 3. Probe A, from HCGJ16, is derived from the 3' end of the first intron. Probe

B is derived from the 5' untranslated region of the cDNA clone HCGJ7. Probe C includes probe B, and extends further in the 3' direction to encompass most of the coding region. Probe D contains the remainder of the coding region, but is located on the 3' side of the internal Eco RI site. Probe A appears to be gene specific, hybridizing with only a single band in each lane. Probes B, C, and D, derived from exons, recognize two major bands in each lane, suggesting the presence of two highly homologous sequences. Somatic cell hybrids (human x mouse) and genomic cloning data confirm the presence of two loci, which map to different chromosomes (Fishman, G. I., L. Rosenthal, T. Shows, and L. A.

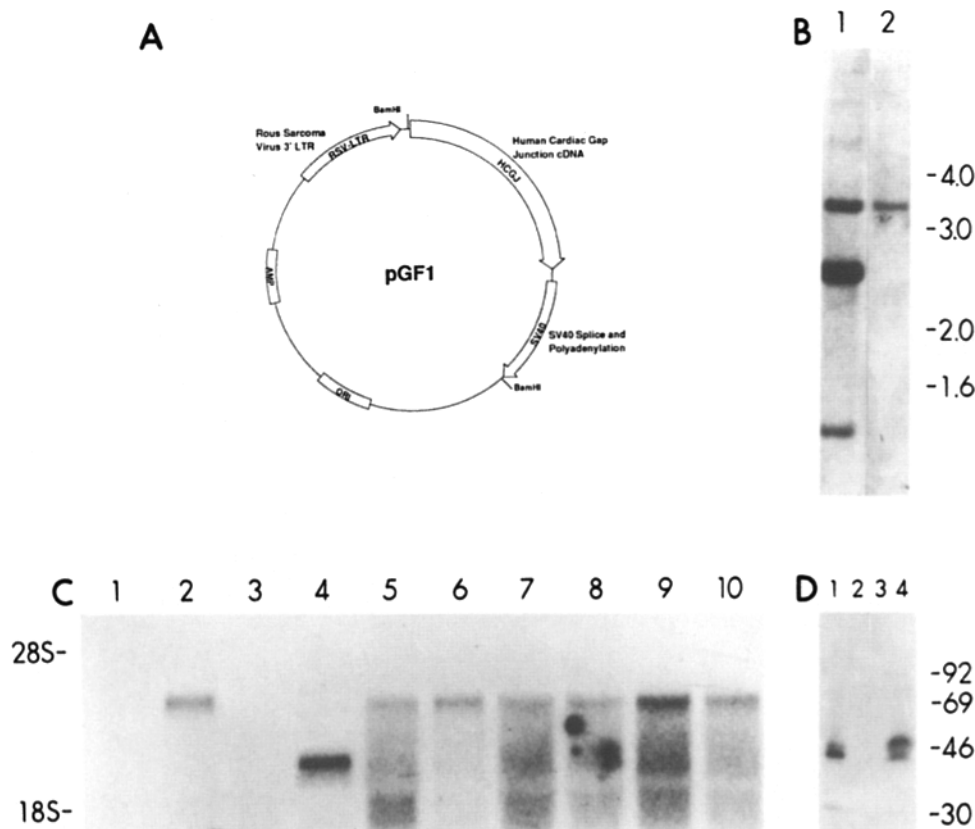


Figure 4. Analysis of the HCGJ transfectant pGF1. (a) Expression vector pGF1. The vector contains nucleotides 1–1,817 of the HCGJ cDNA, the Rous sarcoma virus 3' long terminal repeat (*RSV-LTR*), the 3' SV40 intron and early region polyadenylation site from pSV2Neo (*SV40*), and the origin of replication (*ORI*) and AMP^R cis-tron (*AMP*) from pBR322. The construction is described in the text (see Materials and Methods). (b) Southern blot analysis. Probe C (encompassing bp 1–1,183 of the HCGJ cDNA) was hybridized with Bam HI-digested SKHepl or pGF1 genomic DNA under high stringency conditions. (lane 1) pGF1; (lane 2) SKHepl. Molecular weight markers are indicated. pGF1 DNA contains a new band of 2.8 kb, which is ~10-fold more abundant than the signal from the endogenous HCGJ gene found in both pGF1 and SKHepl cells. The 1.3-kb hybridizing band in lane 1 is probably due to integration of a partial frag-

ment. (c) Northern blot analysis. A cardiac gap junction specific oligonucleotide probe was hybridized under high stringency conditions (see Materials and Methods). (lane 1) Adult rat liver; (lane 2) adult rat heart; (lane 3) nontransfected SKHepl cells; (lane 4) pGF1 cells; (lane 5) human week 20 fetal heart; (lane 6) human week 24 fetal heart; (lane 7) normal adult left ventricle; (lane 8) normal adult right ventricle; (lane 9) myopathic ventricle; (lane 10) myopathic ventricle. The transcript from pGF1 is truncated, as predicted by the expression vector construct. The HCGJ transcript is expressed in fetal, adult, and myopathic hearts, but not in liver or SKHepl cells. (d) Western blot analysis. A polyclonal antibody directed against residues 340–360 of the rat connexin43 protein was incubated with a filter resulting from transfer of total cellular lysates (see Materials and Methods). (lane 1) pGF1; (lane 2) SKHepl; (lane 3) rat liver; (lane 4) rat myocardium. Immunoreactive species of ~43 kD are seen in both pGF1 and rat myocardium, but not nontransfected SKHepl cells or rat liver.

Leinwand, manuscript in preparation). One locus has been cloned from a genomic DNA library and differs from the cDNA by ~4% at the nucleotide level. It appears to be a processed pseudogene, based on the absence of introns and the presence of a poly(A) tail within the genomic sequence. Thus, probe A is specific for the human cardiac gap junction gene, while probes B, C, and D also recognize the pseudogene.

Expression of HCGJ mRNA

To examine the expression of the HCGJ gene in several human cardiac samples, Northern blot analysis was carried out. Shown in Fig. 4 c is an RNA hybridization of 20- and 24-wk human fetal hearts (lanes 5 and 6), the left and right ventricle of a normal adult (lanes 7 and 8), and ventricular tissue from two explanted myopathic hearts (lanes 9 and 10). These limited human samples demonstrate that expression of HCGJ mRNA begins early in cardiac development and persists in both the normal and myopathic adult heart. There appears to be some quantitative variation in expression, but the limited availability of human specimens from developmental stages makes it difficult to precisely define the developmental progression. Also shown is hybridization to rat cardiac RNA (lane 2) which demonstrates that cardiac gap junction

mRNA from both species comigrates at 3.1 kb. No HCGJ mRNA is observed in liver (lane 1).

Functional Expression of the HCGJ Channel

To analyze the functional properties of the human cardiac gap junction channel, we transfected the human hepatoma cell line SKHepl with the expression vector pGF1 (Fig. 4 a) and isolated stable transfectants. One clonal line was selected for further study. Expression of the transfected sequence was confirmed by several approaches, as shown in Fig. 4. To demonstrate chromosomal integration of the expression plasmid, genomic DNA was prepared from the transfectant and digested with Bam HI. This enzyme is predicted to release a 2.8-kb fragment, including the 1,817-bp portion of the HCGJ cDNA, the 900-bp 3'-SV40 fragment, and portions of plasmid polylinker sequence. As shown in Fig. 4 b, hybridization of probe C with genomic DNA shows a prominent band of the expected size (lane 1), which is absent in the parental nontransfected SKHepl cells (lane 2). The relative intensity of this signal, compared to that of the endogenous gene, indicates integration of ~10 copies of the expression plasmid into the genome.

Confirmation of transcription was obtained by Northern

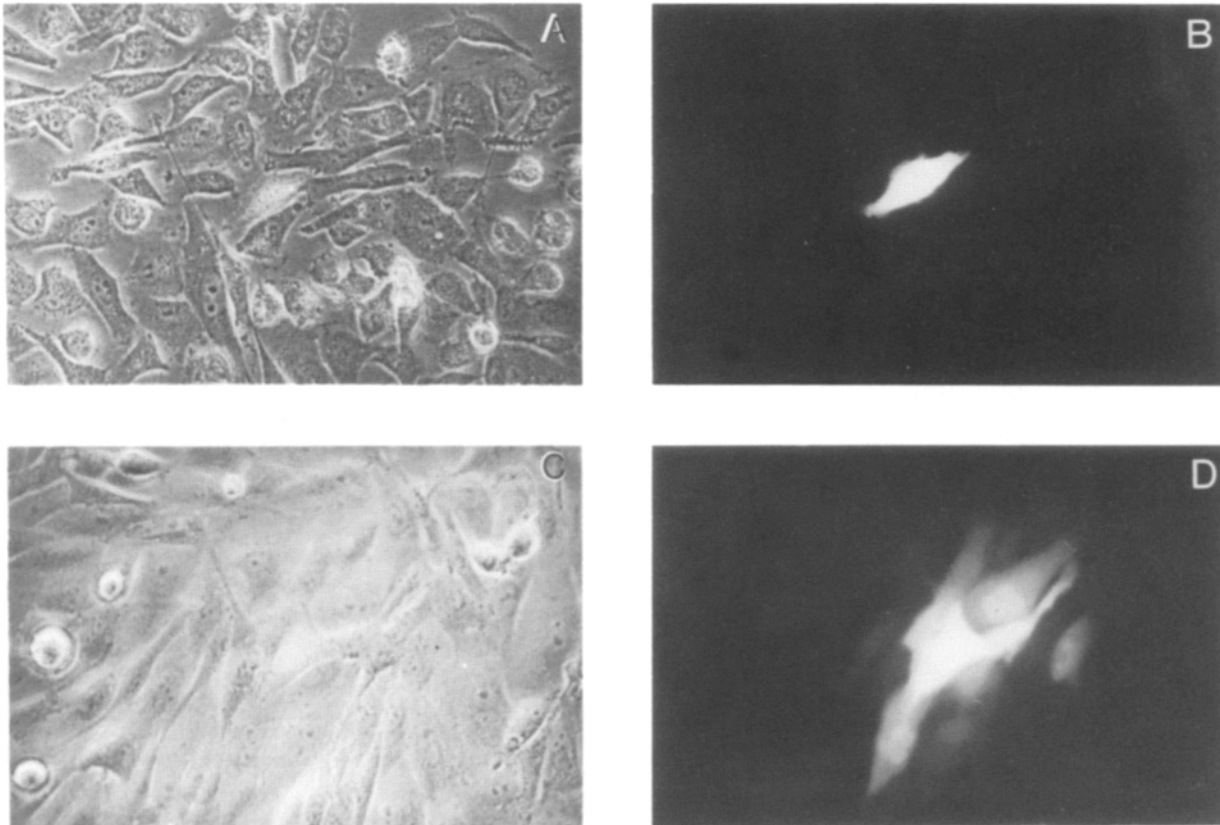


Figure 5. Functional expression of the HCGJ channel. Cells were injected with Lucifer Yellow and phase contrast (*A* and *C*) and fluorescence (*B* and *D*) photomicrographs were obtained 1 min later (see Materials and Methods). *A* and *B* are from nontransfected cells, whereas *C* and *D* are from pGF1. Only pGF1 transfectants show transfer of Lucifer Yellow.

analysis (Fig. 4 *c*). Transcription of pGF1 is driven by the RSV LTR; termination, splicing, and polyadenylation are directed by the 3' SV40 signals. Because the HCGJ fragment is truncated at nucleotide 1,817, the fully processed transcript from pGF1 is predicted to be significantly shorter than that found for the endogenous HCGJ gene. While comigrating bands of 3.1 kb are seen in cardiac tissue from both rat and human (lanes 2 and 5–10), a significantly shorter transcript is seen in the transfectant harboring pGF1 (lane 4). No mRNA for endogenous HCGJ is detectable in the parental SKHepl cells (lane 3).

Western blot analysis (Fig. 4 *d*) was performed to document translation of the HCGJ protein by the pGF1 cell line. Both pGF1 (lane 1) and rat myocardium (lane 4) demonstrate immunoreactive species of ~43 kD. The slight difference in mobility of the major species may be due to differences in phosphorylation state. This is currently under investigation. No immunoreactivity is seen with either the parental SKHepl cell line (lane 2) or rat liver (lane 3).

Direct confirmation of channel assembly and function was demonstrated by both dye transfer and electrophysiological techniques. In Fig. 5, fluorescence microscopy demonstrates the complete absence of Lucifer Yellow dye transfer in nontransfected SKHepl cells (panel *B*). Injection of dye into transfectants leads to rapid transfer of Lucifer Yellow, occasionally reaching third order cells within 1 min (panel *D*). To determine the conductance properties of the HCGJ channel, whole cell voltage clamp recordings were obtained from

cell pairs. Nontransfected SKHepl cells typically display junctional conductance below the level of sensitivity (<20 pS). Infrequently (<10%), a very low level of junctional conductance (<0.6 nS) has been detected between pairs of the parental cells, with unitary conductance <30 pS. The expression of the HCGJ channel increases the conductance markedly (Fig. 6), averaging 18.4 ± 2.31 nS (SE, $n = 15$). Furthermore, the cell coupling is rapidly and reversibly disrupted by halothane, a typical feature of cardiac gap junction channels (Burt and Spray, 1989). After halothane treatment, unitary conductance events were obtained, as seen in Fig. 7. Single-channel conductance for the HCGJ recorded in three cell pairs corresponded to ~60 pS.

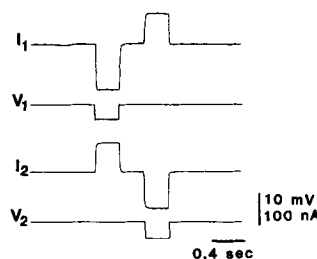


Figure 6. Junctional conductance between pairs of pGF1 cells. Each cell was held at -40 mV and command steps were presented alternately to each cell of the pair. Junctional conductance (g_j) was measured as the current evoked in one cell by the voltage step in the other divided by the amplitude of the voltage step delivered to the other cell (see Materials and Methods). Junctional conductance averaged 18.4 ± 2.31 nS ($n = 15$).

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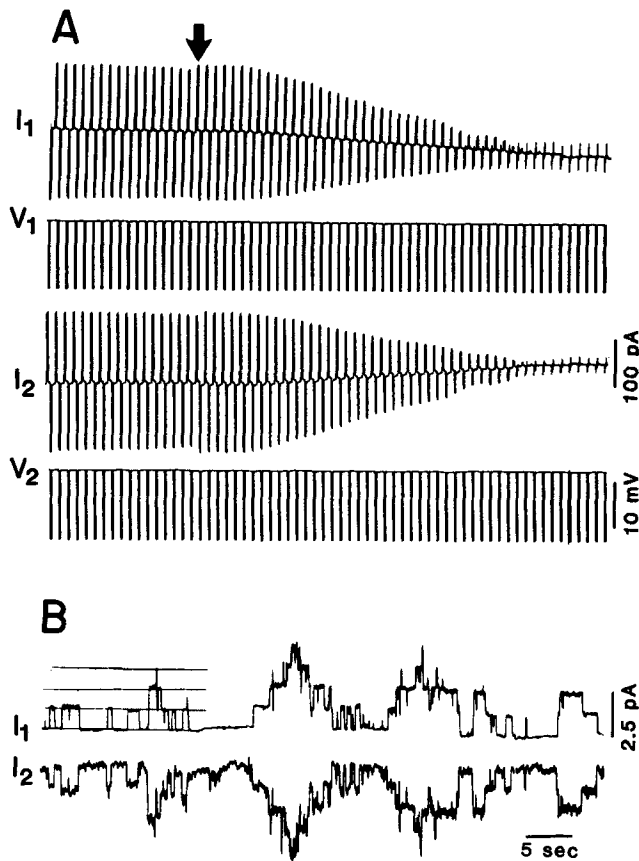


Figure 7. Unitary conductance of the HCGJ channel. (a) Each cell was held at -40 mV and command potentials (V_1 , V_2) were alternately delivered to the two cells. Junctional conductance was initially ~ 10 nS and rapidly declined during exposure of the cell pair to 1.5–2.0 mM halothane (begun at arrow). (b) After cells were almost completely uncoupled, a transjunctional voltage of 23 mV was imposed (upper trace cell held at -14 mV, lower trace cell at -37 mV) and gain on the chart recorder was increased. Discrete events were detectable, corresponding to junctional conductances of 55–60 pS (thin horizontal lines at the start of the record indicate g_j levels of ~ 0 [the initial level], 55, 110, and 165 pS). In this record, simultaneous divergence of the two cells' currents indicate opening of gap junction channels. All records are filtered at 20 Hz; cells were used 3 h after dissociation.

Discussion

This paper describes the molecular characterization and functional expression of the HCGJ channel. The sequence presented for the HCGJ cDNA represents the first full-length sequence for a connexin43 transcript, including the entire 3' untranslated region and poly(A) tail. While previous estimates of cardiac gap junction transcript size were based on electrophoretic mobility (Beyer et al., 1987), our sequence demonstrates that the full-length mRNA is ~ 3.1 kb in size. Interestingly, the major portion of the 3' untranslated region of the human and rat connexin43 sequences diverge completely, despite the marked conservation of sequence which is found comparing human and rat connexin32 (Paul, 1986; Kumar and Gilula, 1986). At the protein level, human connexin43 differs from the rat isoform at nine positions, including the substitution of an alanine for a serine at position 257, and the substitution of a serine for a threonine at posi-

tion 251. Such differences may potentially lead to alterations in patterns of phosphorylation, as discussed below.

The HCGJ protein appears to be the product of a single gene, which was distinguished from a highly homologous processed pseudogene by its specific hybridization with intervening sequence derived from an incompletely processed transcript. The appearance of introns in cDNA libraries has been reported previously, most recently during the isolation of clones for the cystic fibrosis gene (Riordan et al., 1989). The genomic organization of the connexins appears to be well-conserved. The acceptor splice site of the incompletely processed HCGJ transcript, found 16 nucleotides upstream of the initiation codon, is identical to that found in the rat connexin 32 gene (Miller et al., 1988). Similarly, the mouse connexin26 gene contains an intron in the 5' untranslated region (Zhang and Nicholson, 1990). Given the homology that exists between different members of the connexin family, it is possible that the various isoforms evolved through gene duplication and subsequent divergence, in response to tissue-specific physiologic requirements. Despite this homology, the HCGJ gene (human connexin43) and human connexin32 gene (as well as a connexin43-processed pseudogene), all map to different chromosomes (Fishman, G. I., L. Rosenthal, T. Shows, and L. A. Leinwand, manuscript in preparation).

An essential property of channels in biological membranes is that they are gated by biological and pharmacological stimuli, such that the equilibrium between open and closed states can be shifted. For cardiac gap junction channels, treatments that gate include intracellular pH, second messengers including cyclic nucleotides, and lipophilic molecules such as halothane (Burt and Spray, 1988a,b, 1989). Isolated rat cardiocyte pairs, for example, show increased junctional conductance in response to agents which elevate intracellular cAMP (Burt and Spray, 1988b), and in response to diacylglycerol and phorbol ester, which activate kinase C (Spray and Burt, 1990). Decreased junctional conductance in rat cardiocyte pairs occurs in response to agents which elevate intracellular cGMP (Burt and Spray, 1988b). However, the predicted HCGJ amino acid sequence does not reveal consensus sites for modification by PKA or cGMP-dependent protein kinase. This is in contrast to connexin32 from rat hepatocytes, which is phosphorylated by PKA, in concert with an elevation in junctional conductance (Saez et al., 1986), and which (along with human connexin32) demonstrates well-recognized PKA modification sites (Kumar and Gilula, 1986). Thus, cyclic nucleotide dependent phosphorylation of connexin43 may utilize cryptic sites or involve indirect mechanisms.

An additional determinant of gap junction conductance is intracellular pH. Gating of junctional channels may be mediated through conformational changes related to titration of specific histidine residues in the cytoplasmic loop (Spray and Burt, 1990). The HCGJ cDNA sequence predicts the presence of two histidines in this portion of the HCGJ protein.

Xenopus oocytes have been used previously to compare functional properties of cloned connexins (Dahl et al., 1987; Swenson et al., 1989; Werner et al., 1989). However, the oocyte expresses at least one endogenous connexin (Ebihara et al., 1989; Gimlich et al., 1990), thereby complicating interpretation of the data. In addition, the large size of the oocyte prevents single channel recordings of the exogenous

channels. These limitations are overcome by the use of stably transfected communication deficient mammalian cell lines, as reported here.

The parental cell line used for our transfection studies, SKHepl, is deficient in intercellular communication, as evidenced by failure of cell to cell transfer of Lucifer Yellow (Eghbali et al., 1990; current study, Fig. 5). SKHepl cells have no detectable mRNA for either connexin32, as determined by Northern blot analysis (Eghbali et al., 1990) or connexin43 mRNA, as determined by Northern blot analysis (current study, Fig. 4) and S1 protection analysis (data not shown). The low level of junctional conductance which is infrequently recorded between cell pairs appears to be due to an unidentified connexin isoform, with a unitary conductance value of <30 pS (Moreno, A. P., B. Eghbali, and D. C. Spray, manuscript in preparation). Although other cell lines have been reported to be communication incompetent, high resolution recording or substrate manipulation, e.g., cAMP exposure, has revealed substantial junctional conductance and dye permeability (Loewenstein, 1979). SKHepl cells do not respond to cAMP, and are therefore unique at present in exhibiting extremely low and well defined levels of endogenous gap junction expression. Furthermore, whole cell recordings on these transfectants permit the unambiguous assignment of channel properties such as unitary conductance, which are inaccessible in the *Xenopus* oocyte expression system.

The current study provides the first functional analysis of the human cardiac gap junction channel. We find that junctional conductance between cell pairs expressing the HCGJ channel is high, presumably reflecting the abundant expression of the connexin mRNA and protein, rather than a property of the channel per se. This is consistent with our hybridization studies, which demonstrate incorporation of multiple copies of the HCGJ cDNA into the genome. One property that appears to characterize connexins in different tissues is that of size of the unitary conductance event. In cardiac myocytes of rat and guinea pig, the dominant unitary conductance observed between low conductance cell pairs is ~50–60 pS (Rook et al., 1988; Rudisuli and Weingart, 1989; Burt and Spray, 1988a), a value similar to that reported here for the human cardiac gap junction channel. In contrast, a unitary conductance of 120–150 pS is recorded from systems in which the dominant gap junction protein is connexin32, including expression systems as diverse as lipid bilayers, pairs of acinar cells and transfected SKHepl cells (e.g., Neyton and Trautman, 1985; Spray et al., 1986; Young et al., 1987; Eghbali et al., 1990). Thus, it appears evident that connexin type is an essential determinant of the unitary conductance of gap junction channels.

The precise functional domains of gap junction proteins involved in modulating conductance, including sensitivity to pH, voltage, and second messenger molecules, are the subject of considerable investigation. As additional clones for connexin proteins become available, sequence analysis should facilitate the recognition of important functional domains. Based on these assignments, specific mutant constructs can be designed and analyzed in a similar manner. Such studies undoubtedly will help us more fully understand those primary structural features of gap junction proteins which provide specific functional attributes.

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