

# Connexin43: A Protein from Rat Heart Homologous to a Gap Junction Protein from Liver

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**Abstract.** Northern blot analysis of rat heart mRNA probed with a cDNA coding for the principal polypeptide of rat liver gap junctions demonstrated a 3.0-kb band. This band was observed only after hybridization and washing using low stringency conditions; high stringency conditions abolished the hybridization. A rat heart cDNA library was screened with the same cDNA probe under the permissive hybridization conditions, and a single positive clone identified and purified. The clone contained a 220-bp insert, which showed 55% homology to the original cDNA probe near the 5' end. The 220-bp cDNA was used to rescreen a heart cDNA library under high stringency conditions, and three additional cDNAs that together spanned 2,768 bp were isolated. This composite cDNA contained a single 1,146-bp open reading frame coding for a predicted polypeptide of 382 amino acids with a molecular mass of 43,036 D. Northern analysis of various rat tissues using this heart cDNA as probe showed hybridization to 3.0-kb bands in RNA isolated from heart, ovary, uterus, kidney, and lens epithelium.

Comparisons of the predicted amino acid sequences for the two gap junction proteins isolated from heart and liver showed two regions of high homology (58 and 42%), and other regions of little or no homology. A model is presented which indicates that the conserved sequences correspond to transmembrane and extracellular regions of the junctional molecules, while the nonconserved sequences correspond to cytoplasmic regions. Since it has been shown previously that the original cDNA isolated from liver recognizes mRNAs in stomach, kidney, and brain, and it is shown here that the cDNA isolated from heart recognizes mRNAs in ovary, uterus, lens epithelium, and kidney, a nomenclature is proposed which avoids categorization by organ of origin. In this nomenclature, the homologous proteins in gap junctions would be called connexins, each distinguished by its predicted molecular mass in kilodaltons. The gap junction protein isolated from liver would then be called connexin32; from heart, connexin43.

**G**AP junctions are composed of collections of membrane channels, called connexons, which join in mirror symmetry with connexons in the membrane of the adjacent cell. These pairs of connexons permit the intercytoplasmic exchange of small metabolites and ions between cells. Each connexon is composed of a hexamer of an integral membrane protein, whose complete cDNA has been cloned from rat and human liver, with a predicted molecular mass of 32 kD (23, 32). The mRNA coding for this protein is not unique to the liver, but may be detected in other, but not all, organs within the same animal (32). In this paper, we show that a related mRNA is found in abundance in heart and other organs, and that mRNAs coding for both the liver and heart gap junction proteins are in some cases detected in the same organ.

Thus, these gap junction mRNAs are not confined to the organs in which they were first observed, necessitating a nomenclature system which avoids mention of source. We propose reintroduction of the name connexin as a generic

term. Since the observed electrophoretic mobilities for connexin proteins enriched from both liver and heart vary according to experimental conditions, we propose the use of the predicted molecular mass in combination with the generic term in order to distinguish between different members of the connexin family. For example, the liver gap junction protein would be referred to as connexin32, and, as a result of the data presented in this paper, the heart protein as connexin43.

Published studies suggest that there are related, but nonidentical, connexins in liver and heart. Antisera raised in several laboratories using connexin32 as antigen exhibit different tissue specificities (9, 21, 31, 45). In one case, the antiserum recognizes structures at myocardial intercalated discs by immunofluorescence, and corresponding Western blots identify an immunoreactive polypeptide (21). In our laboratory, a similar antiserum fails to recognize either the structure or the polypeptide in heart (31). An antiserum that was raised against a synthetic oligopeptide derived from the

NH<sub>2</sub>-terminal sequence of connexin32 by Zervos et al. (45) recognizes immunoreactive peptides in homogenates of liver, heart, and uterus. Taken together, these results suggest that connexin32 and the immunoreactive polypeptide in the heart share some antigenic determinants, but also contain unique structure. Independent evidence for this conclusion was obtained by NH<sub>2</sub>-terminal sequence analysis of proteins found in gap junction preparations. Nicholson et al. (30) and Manjunath et al. (27) have shown that a 44–47-kD polypeptide from myocardium is 43% homologous to connexin32 over the first 28 amino acids. Low stringency Northern blot analysis (32) provides additional support for the existence of a molecule in myocardium related to connexin32, and provides the basis for the screening strategy used in this paper to clone a unique connexin cDNA.

## Materials and Methods

### RNA Isolation and Northern Blots

RNA was isolated by homogenization of freshly dissected rat organs in guanidine isothiocyanate followed by centrifugation through CsCl (6). Uteri were obtained from 20–22-d pregnant rats and ovarian tissue from 21-d-old females primed for two successive days before sacrifice with 10 IU intraperitoneal injections of pregnant mare's serum gonadotropin (PMSG;<sup>1</sup> Sigma Chemical Co., St. Louis, MO) in PBS. Previous studies have shown that gap junction structures are abundant in these two tissues (1, 15). For whole lens mRNA isolation, lenses were dissected from 72 rat eyes and directly homogenized in guanidine. While care was taken to dissect the lenses free from adherent ciliary epithelium, electron microscopy of similarly dissected lenses frequently revealed a tightly adherent layer of ciliary epithelium cells adjacent to the lens capsule, unobservable in the dissecting microscope (data not shown). To remove these adherent cells, a second preparation of mRNA was made from lenses digested first with 0.1% trypsin after dissection, as described by FitzGerald and Goodenough (12), then homogenized in guanidine. Finally, a third preparation of mRNA was made from 72 rat eyes that were first dissected and then had their capsules mechanically removed, a procedure which also removes the lens epithelium (28).

RNA samples (10 µg) were subjected to electrophoresis on 1% agarose/formaldehyde gels and capillary blotted onto nylon membranes (Hybond-N; Amersham Corp., Arlington Heights, IL) as described by Davis et al. (8). RNA was cross-linked to the membrane by exposure to a medium wavelength (300 nm) UV transilluminator for 5 min. Radiolabeled cDNA probes were prepared by isolation of the DNA fragments by electrophoresis in low melting-temperature agarose and labeling using the Klenow fragment of DNA polymerase I and hexanucleotide primers as described by Feinberg and Vogelstein (11). Low stringency blots were prehybridized in 0.75 M Na<sub>2</sub>HPO<sub>4</sub> (pH 7.2), 5% SDS, 100 µg/ml salmon sperm DNA for 1 h at 55°C and then hybridized overnight in the same buffer with the labeled probe at 55°C. Blots were then washed in 0.3 M Na<sub>2</sub>HPO<sub>4</sub>, pH 7.2, 1% SDS once at room temperature and three times at 55°C over 1 h before exposure to Kodak XAR-5 film at –80°C with an intensifying screen. High stringency blots were prehybridized and hybridized in the same solutions at 65°C and washed in 0.015 M Na<sub>2</sub>HPO<sub>4</sub>, 1% SDS at 65°C before autoradiography. Hybridization and washing were carried out in Hybrid-Ease chambers (Hoefer Scientific Instruments, San Francisco, CA).

### cDNA Library Construction and Screening

Poly(A) RNA was isolated from total rat heart RNA by chromatography on oligo-dT cellulose (3). cDNA was synthesized by a modification of the method of Gubler and Hoffman (17) using the kit supplied by Amersham Corp. After blunting with T4 DNA polymerase and methylation with Eco RI methylase, kinased Eco RI linkers (New England Biolabs, Beverly, MA) were ligated to the cDNA with T4 DNA ligase. The linkered cDNA was digested with Eco RI, and the high molecular mass cDNA was separated from excess linkers by gel filtration. The cDNA was ligated to Eco RI-

digested, dephosphorylated lambda gtlI arms (Promega Biotec, Madison, WI) and packaged using extracts prepared by Stratagene (San Diego, CA). This library contained ~10<sup>6</sup> recombinants with an average insert size of 1.1 kb. A second rat heart cDNA library was obtained commercially (Clontech, Palo Alto, CA).

The synthesized cDNA library was screened by hybridization of nitrocellulose filter plaque lifts in 5× SSC (1X SSC is 150 mM NaCl, 15 mM Na citrate, pH 7.0.), 1% SDS, 100 µg/ml salmon sperm DNA with the <sup>32</sup>P-labeled rat liver gap junction cDNA overnight at 50°C, followed by three washes, 20 min each, in 2X SSC, 1% SDS at 50°C before exposure to Kodak XAR-5 film. The rat heart cDNA clone (D7) isolated by this low stringency procedure was used to rescreen this library and the Clontech library by hybridization and washing in the same solutions, but at 65°C.

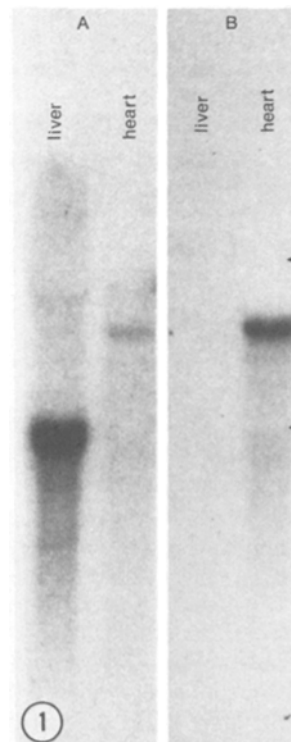
### DNA Sequence Analysis

Lambda clones were purified from plate lysates by DEAE cellulose chromatography (19). The cDNAs were released by Eco RI digestion and subcloned into the Eco RI site of the plasmid Bluescript (Stratagene). Ordered sets of overlapping deletions were constructed by the exonuclease III/S1 nuclease procedure of Henikoff (20) as modified by Lawler and Hynes (25). Single-stranded DNA was isolated by culturing Bluescript transformants in *Escherichia coli* strains JM101 or XL1-B (Stratagene) with the helper phage R408 (Stratagene) as described by the supplier. All sequencing was performed by the chain termination method of Sanger et al. (36) using this single-stranded template and dideoxy sequencing reagents as described by Williams et al. (42), except that 7-Deaza-dGTP (Boehringer Mannheim Biochemicals, Indianapolis, IN) was used in place of dGTP in equimolar amounts. All clones described were completely sequenced in both directions. Sequence data was analyzed using computer programs from Intelligenetics (Palo Alto, CA) and International Biotechnologies (New Haven, CT). Database searches were conducted by the staff of the Molecular Biology Computer Research Resource of the Dana-Farber Cancer Institute.

## Results

### Northern Blots and Isolation of cDNA Clones

Previous low stringency Northern blots suggested that the cDNA for connexin32 hybridized to a message of ~1.2 kb



**Figure 1.** Northern blot analysis. (A) Low stringency hybridization of coding portions of the rat liver gap junction cDNA to total RNA from rat liver and heart. Each lane contains 10 µg RNA. Hybridization is to a 1.6-kb band in liver and a 3.0-kb band in heart. (B) High stringency hybridization of rat heart gap junction cDNA probe (G2) to the same blot as in A after removal of liver probe by boiling in distilled water. The heart probe hybridizes to a band of mobility identical to that seen with the liver probe. Arrowheads indicate positions of 28S and 18S rRNA subunits.

1. Abbreviation used in this paper: PMSG, pregnant mare's serum gonadotropin.

in rat heart RNA (32). Further studies suggested that such hybridization was due to noncoding portions of the connexin32 cDNA (data not shown). Connexin32 cDNA was digested with Bgl I and Eco RI to isolate a fragment containing bases 1-848, which made up 95% of the coding region and none of the 3' untranslated sequence. This fragment hybridized to a single band of 3.0 kb on Northern blots of rat heart RNA at low stringency (Fig. 1 A).

We used the connexin32 cDNA fragment to screen 150,000 bacteriophage plaques from the rat heart cDNA library under similar low stringency conditions. One consistently positive clone was isolated. This cDNA clone (D7, Fig. 2) contained 220 bp, and its cDNA sequence showed 55% homology to a region near the 5' end of connexin32 cDNA. The amino acid sequence predicted by an open reading frame in this cDNA showed a similar high level of amino acid homology to connexin32. No further clones were isolated from this library. Clone D7 was used as hybridization probe to screen a second rat heart library (Clontech) under high stringency conditions, and three longer clones (G1, 2.5 kb; G2, 1.4 kb; and G3, 1.3 kb) were isolated. The cDNA inserts were oriented and aligned by restriction mapping (Fig. 2). All three of these cDNA inserts hybridized to a single 3.0-kb band on Northern blots of rat heart RNA (at high stringency). Hybridization with the probe G2 is shown in Fig. 1 B. The 3.0-kb band is indistinguishable in mobility from the band identified at low stringency with the connexin32 cDNA fragment.

### Nucleotide and Amino Acid Sequence

The nucleotide sequences of all clones were determined; they overlapped with no discrepancies. The composite cDNA sequence (Fig. 3) contains 2,768 nucleotides. The first ATG initiation codon occurs at base 202 and is followed by an open reading frame of 1,146 bases and a TAA termination codon at position 1,348. The reading frame is closed near the 5' side of this presumed initiation codon. The coding region is followed by 1,218 bases of 3' untranslated sequence, which contains multiple termination codons in all three frames, but no polyadenylic acid tail.

The cDNA sequence predicts a polypeptide containing 382 amino acids (Fig. 3) with a calculated molecular mass

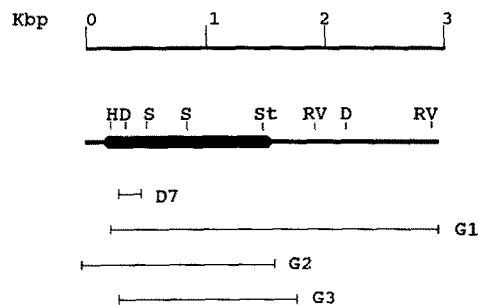


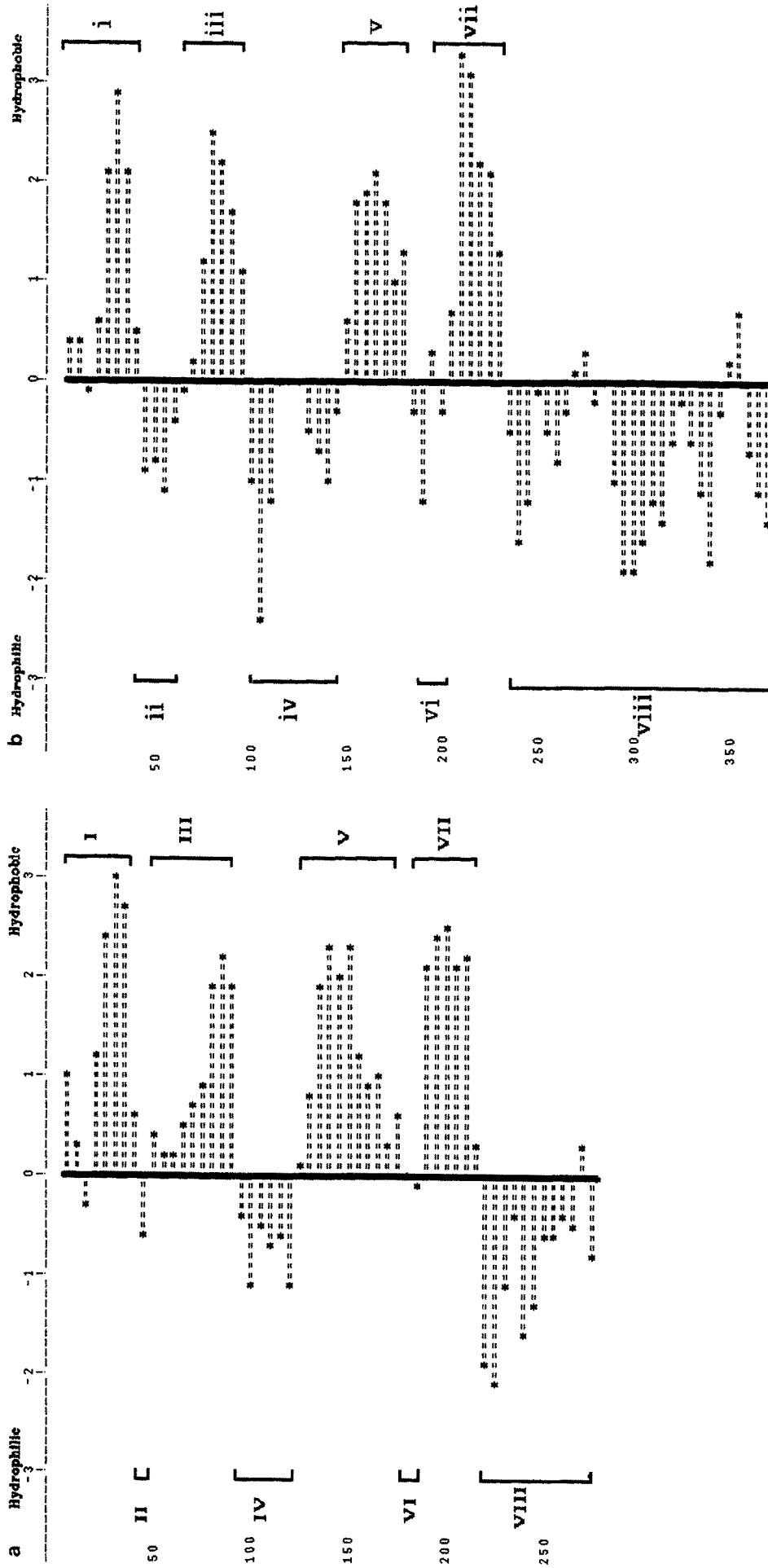
Figure 2. Alignment and restriction map of heart gap junction cDNA clones. The coding region of the cDNA is represented by a heavy line, while 5' and 3' untranslated regions are represented by a thinner line. Restriction sites are those for Hind III (H), Dra II (D), Sac I (S), Stu I (St), and Eco RV (RV). Clone D7 is the 220-bp cDNA isolated by low stringency screening of the heart cDNA library with the liver gap junction cDNA probe. Clones G1, G2, and G3 were isolated by rescreening with D7 as probe.

GGCTTTACG	AGGTATCAG	ACTTTCCTT	CATTGGGGG	AAGGCTGAG	GAAAGTACC	AACAGCAGCA	70
GGATTTCCAC	CAACTTGGC	GGCCTTCCA	CTTTCATTA	GTGAAGAGA	GGTCCCCAGA	AGCCTCCAA	140
ATG GGT GAC TGG AGT GCG TTG GGG	AGG CTT CTG GAC AGG GTC GAA GCC TAC TCC ACC GCT	261					
MET Gly Asp Trp Ser Ala	Leu Gly Lys Leu Leu Asp Lys Val Glu Ala Tyr Ser Thr Ala						
GGA GGG AAG GTG TGG CTT GCA GTG	CTC TTC ATA TTC AGA ATT CTG CTC CTG GGG ACA GCT	321					
Gly Gly Lys Val Trp Leu Ser Val	Leu Phe Ile Phe Arg Ile Leu Leu Gly Thr Ala						
GTT GAG TCA GCT TGG GGT GAT GAA	GAG TCT GCC TTT CGC TGT AAC ACT GAA CAA CCT GGC	381					
Val Glu Ser Ala Trp Gly Asp Glu	Gln Ser Ala Phe Arg Cys Asn Thr Glu Gln Pro Gly						
TGC GAA AAC GTC TGC TAT GAC AAG	TCC TTC CCC ATC TCT CAG CTG GCG TTC TGG GTC CTT	441					
Cys Glu Asn Val Phe Val Tyr Asp Lys	Ser Phe Lys Ser Phe Ile Leu Val Phe Thr Tyr Val Met						
CAG ATC ATA TTC GTG TCT GTG CCC	ACC CTC CTG TAC TTG GCC CAT GTG TTC TAT GTG ATG	501					
Val Arg Thr Val Ser Val Pro Thr	Thr Leu Leu Glu Glu Leu Lys Val Val Thr Tyr Val Met						
AGG AAG GAA GAG AGC ATA AAC AAG	AAA GAA GAG GAG CTC AAG GTA GGC CAG ACT GAC GGG	561					
Arg Lys Glu Asn Val Phe Val Tyr Lys	Leu Asn Lys Lys Glu Glu Leu Lys Val Thr Arg Thr Arg Glu						
GTG AAG GTG GAG ATG CAG CTG AAG	CAG ATT GAA ATC AAG AAG TTC AAG TAC GGG ATT GAA	621					
Val Asn Val Glu MET His Lys Leu Lys	Gln Ile Glu Ile Lys Lys Thr Tyr Val Thr Arg Thr Val Met						
GAG CAG GGC AAG GTC AAG ATG AGG	GGC GGC TTG CTG AGA ACC TAC ATE ATE ACC ATE CTC	681					
Phe Lys Ser Phe Val Lys Met Arg Gly	Gly Leu Leu Ile Phe Ile Glu Thr Tyr Ile Thr Arg Thr Arg Glu						
TTC AAG TCT GTC TTC CAG GTG GCC	TTC CTG CTC ATC CAG TGG TAC ACT TAT GGG TTC AGC	741					
Leu Ser Asp Val Phe Glu Val Ala Phe	Leu Leu Ile Glu Thr Tyr Ile Thr Tyr Ile Thr Arg Thr Arg Glu						
TTG AGC GCG GTC TAC ACC TGC AAG	AGA GAT CCC TGC CCC CAG CAG GTA GAC TGC TTC CTC	801					
Lys Ser Arg Thr Thr Cys Lys Arg Asp	Lys Arg Asp Thr Thr Lys Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr						
TCA CGT CCC ACG GAG AAA ACC ATC	TTC ATC ATC TTC ATG CTG GTG TCC TTG GTG TCT	861					
Ser Arg Thr Thr Glu Lys Thr Ile Phe	Ile Phe Ile Ile Phe Ile Phe Ile Phe Ile Phe Ile Phe Ile Phe Ile Phe						
CTC GCT TTG AAC ATC ATT GAG CTC	TTC TAC GTC TTC TTC AAA GGC GTT AAG GAT CCG GTG	921					
Leu Ala Leu Asn Ile Ile Glu Leu Phe	Tyr Val Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr						
AAG GGA AGA AGC GAT CCT TAC CAC	GCC ACC ACT GGC CCA CTG AAG CCA TCA AAA GAC TGC	981					
Lys Glu Lys Arg Ser Asp Pro Tyr His	Ala Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr						
GGA TCT CCA AAA TAC GGC TAC TTC	AAT GGC TGC TCC TCA CCA ACC GCT CCA CTC TCG CCT	1041					
Gly Tyr Ser Pro Lys Tyr Ala Tyr Phe	Asn Gly Lys Ser Ser Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr						
ATG TCT CCT CCT GGG TAC AAG CTG	GTT ACT GGT GAC AGA AAC AAT TCC TCG TGC CGC AAT	1101					
Met Thr Ser Pro Gly Tyr Lys Leu Val	Thr Gly Asp Arg Asn Ser Ser Ser Ser Ser Ser Ser Ser Ser Ser Ser						
TAC AAC AAG CAA GCT ACC GAG CAA	AGC TGG GCG AAC TAC ACC GCA GAG CAA AAT CGC ATG	1161					
Val Asn Lys Glu Ala Ser Glu Gln Asn	Trp Ala Glu Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr						
GGG CAG GCC GGA AGC ACC ATC TCC	AAC TGC CAC GCC CAG CCG TTC TAT TCC CCC GAC GAC	1221					
Gly Ser Arg Thr Thr Ile Ser Asn Ser	Asn Ser Asn Ser Asn Ser Asn Ser Asn Ser Asn Ser Asn Ser Asn Ser Asn						
AAC CAG AAT GCC AAA AAA GTT GCT	GCT GGA CAT GAA CTC GAG CCA TTA GCC ATC GTG GAC	1281					
Asn Glu Asn Ala Lys Lys Val Ala Ala	Ala Glu His Glu Leu Leu Ala Ile Val Asp Leu						
CAG CGA CCT TCC AGC AGA GCC AGC	AGC GGC GCC AGC AGC GCT CGG CCT GAT GAC CTG	1341					
Gln Arg Pro Ser Ser Arg Ala Ser Ser	Arg Ala Ser Ser Arg Ala Ser Ser Arg Pro Arg Pro Asp Asp Leu						
GAG ATT TAA	ACCGGCTTGA GCATCGAGCT GTCGATTATG GAGGAGAAAA AAGAGTGCTT	1400					
Glu Ile							

Figure 3. Sequence of rat heart gap junction cDNA. The complete nucleotide sequence constructed from the overlapping sequences of clones D7, G1, G2, and G3 is shown with residues numbered on the right side. The derived amino acid sequence is shown with residues numbered on the left. This predicted protein has a molecular mass of 43,036. The available partial protein sequence matches amino acids 2-20 of the predicted sequence.

of 43,036, a size similar to the major polypeptide of 44,000-47,000 which Manjunath et al. (26) have characterized in cardiac gap junction preparations. Confirmation that this represents the sequence of a heart gap junction protein comes from the close match between the published NH<sub>2</sub>-terminal amino acid sequence of a rat heart gap junction protein determined by Nicholson et al. (30) and Manjunath et al. (27) and residues 2-20 of the predicted sequence shown here. The only difference is that those authors were unable to tell whether residue 2 was glycine, alanine, or histidine. The predicted residue is glycine. The predicted sequence also contains a single methionine residue preceding the first residue in the mature protein. Presumably, this methionine is proteolytically removed posttranslationally to leave a mature protein of 42,867 D. There is no evidence for a longer leader or signal sequence. Following the suggestion on nomenclature presented in the introduction, this protein will be called connexin43.

Connexin43 is predicted to be very basic (pI = 10.19), similar to the pI of 10.88 of connexin32. At neutral pH connexin43 would have 13.9% basic, 9.4% acidic, 34.3% polar, and 42.4% nonpolar residues. With 53 basic residues



CONNEXIN43	1	<b>M</b> GD <b>S</b> A <b>L</b> G <b>K</b> L <b>D</b> K <b>Q</b> A <b>S</b> T <b>A</b> G <b>K</b> W <b>L</b> S <b>V</b> L <b>F</b> I <b>F</b> R <b>L</b> L <b>L</b> L <b>S</b> T <b>V</b> E <b>S</b> A <b>W</b> G <b>D</b> E <b>Q</b> S <b>A</b> P <b>R</b> E <b>N</b> T <b>Q</b> P <b>G</b> C <b>E</b> N <b>V</b> C <b>Y</b> D <b>K</b> S <b>F</b> P <b>I</b> S <b>H</b> V	75
CONNEXIN32	1	<b>M</b> N <b>I</b> P <b>G</b> L <b>V</b> T <b>L</b> L <b>S</b> G <b>N</b> R <b>H</b> E <b>T</b> A <b>G</b> R <b>V</b> L <b>S</b> V <b>L</b> F <b>I</b> F <b>R</b> I <b>M</b> V <b>L</b> V <b>A</b> E <b>S</b> W <b>G</b> D <b>E</b> R <b>S</b> E <b>N</b> T <b>Q</b> P <b>G</b> C <b>N</b> S <b>V</b> C <b>Y</b> D <b>H</b> F <b>F</b> I <b>S</b> H <b>V</b>	74
CONNEXIN43	76	<b>R</b> F <b>M</b> L <b>Q</b> L <b>I</b> F <b>V</b> S <b>M</b> H <b>L</b> L <b>L</b> Y <b>L</b> A <b>H</b> V <b>F</b> Y <b>V</b> M <b>R</b> K <b>E</b> E <b>K</b> L <b>N</b> K <b>K</b> E <b>E</b> L <b>K</b> V <b>A</b> Q <b>T</b> D <b>C</b> V <b>N</b> V <b>E</b> M <b>H</b> L <b>K</b> Q <b>E</b> L <b>R</b> K <b>F</b> K <b>Y</b> G <b>I</b> E <b>E</b> L <b>K</b> V <b>K</b> M <b>R</b> G	150
CONNEXIN32	75	<b>R</b> L <b>S</b> L <b>Q</b> L <b>L</b> I <b>V</b> S <b>T</b> E <b>L</b> L <b>V</b> A <b>M</b> H <b>V</b> A <b>H</b> Q <b>H</b> E <b>K</b> K <b>M</b> ----- <b>L</b> R <b>L</b> ----- <b>R</b> G <b>H</b> D <b>P</b> L <b>H</b> L <b>E</b> ----- <b>H</b> - <b>K</b> V <b>H</b> I <b>S</b> G <b>T</b>	130
CONNEXIN43	151	<b>L</b> L <b>R</b> T <b>V</b> L <b>S</b> L <b>I</b> L <b>F</b> K <b>S</b> V <b>F</b> E <b>V</b> A <b>P</b> L <b>-</b> L <b>I</b> - <b>Q</b> W <b>I</b> L <b>-</b> E <b>G</b> F <b>S</b> L <b>S</b> A <b>V</b> T <b>C</b> K <b>R</b> D <b>P</b> C <b>H</b> Q <b>V</b> D <b>C</b> F <b>L</b> S <b>R</b> P <b>T</b> E <b>K</b> T <b>L</b> L <b>I</b> L <b>F</b> M <b>L</b> V <b>V</b> S <b>L</b> S <b>L</b> A	222
CONNEXIN32	131	<b>L</b> W <b>V</b> T <b>V</b> L <b>S</b> V <b>V</b> E <b>R</b> L <b>L</b> F <b>E</b> A <b>V</b> E <b>M</b> Y <b>V</b> F <b>L</b> L <b>P</b> G <b>A</b> M <b>-</b> V <b>R</b> L <b>I</b> - <b>K</b> E <b>A</b> F <b>P</b> C <b>H</b> T <b>V</b> D <b>C</b> F <b>S</b> R <b>P</b> T <b>E</b> K <b>T</b> M <b>E</b> T <b>V</b> E <b>L</b> A <b>S</b> G <b>I</b> C <b>I</b> T	203
CONNEXIN43	223	<b>L</b> N <b>I</b> L <b>L</b> E <b>L</b> F <b>V</b> F <b>F</b> K <b>G</b> V <b>D</b> R <b>V</b> K <b>G</b> R <b>S</b> D <b>P</b> Y <b>H</b> A <b>T</b> T <b>G</b> F <b>L</b> S <b>P</b> S <b>K</b> D <b>C</b> G <b>S</b> P <b>K</b> Y <b>A</b> Y <b>F</b> N <b>G</b> C <b>S</b> S <b>P</b> T <b>A</b> F <b>L</b> S <b>P</b> M <b>S</b> P <b>P</b> G <b>Y</b> K <b>L</b> V <b>T</b> G <b>R</b> N <b>N</b> S	297
CONNEXIN32	204	<b>L</b> N <b>V</b> A <b>E</b> V <b>V</b> L <b>L</b> I <b>R</b> A <b>C</b> A <b>R</b> A <b>Q</b> R <b>R</b> S <b>N</b> P <b>S</b> R <b>K</b> G <b>S</b> G <b>F</b> G <b>H</b> R <b>L</b> S <b>P</b> E <b>Y</b> K <b>Q</b> E <b>I</b> N <b>K</b> L <b>L</b> S <b>E</b> D <b>G</b> S <b>L</b> K <b>D</b> I <b>L</b> R <b>R</b> S <b>P</b> G <b>T</b> G <b>A</b> L <b>E</b> K <b>S</b> D	278
CONNEXIN43	298	<b>C</b> R <b>N</b> V <b>N</b> Q <b>A</b> S <b>E</b> Q <b>N</b> W <b>A</b> N <b>Y</b> S <b>A</b> E <b>Q</b> N <b>R</b> M <b>G</b> Q <b>A</b> G <b>S</b> T <b>I</b> S <b>N</b> S <b>H</b> A <b>Q</b> P <b>F</b> D <b>P</b> D <b>D</b> N <b>Q</b> A <b>K</b> R <b>V</b> A <b>A</b> G <b>H</b> E <b>L</b> Q <b>L</b> A <b>I</b> V <b>D</b> Q <b>R</b> P <b>S</b> R <b>A</b> S <b>S</b> R <b>A</b> S	372
CONNEXIN32	279	<b>R</b> C <b>S</b> A <b>C</b>	283
CONNEXIN43	373	<b>S</b> R <b>P</b> R <b>D</b> D <b>L</b> E <b>I</b>	382

Figure 5. Comparison of amino acid sequences of connexin43 and connexin32. The connexin43 and connexin32 sequences were optimally aligned so as to match identical residues, which are shown enclosed in boxes. Dashes indicate gaps introduced to optimize alignment. 58% of residues 1-105 of connexin43 are identical to their counterparts in residues 1-104 of connexin32. 42% of residues 142-246 in connexin43 are identical to their counterparts in residues 122-227 of connexin32. The middle and COOH-terminal regions of the molecules show little homology.

(including 8 histidines) and 36 acid residues it would have a net positive charge of 17. There are three potential sites for N-linked glycosylation (consensus Asn-X-Ser/Thr) at residues 295-297, 296-298, and 312-314.

The amino acid sequence was analyzed by the procedure of Kyte and Doolittle (24), which predicts the hydrophobic nature of local regions in the sequence (Fig. 4 b). There are four major hydrophobic regions (labeled i, iii, v, and vii), alternating with four hydrophilic regions (labeled ii, iv, vi, and viii). Region iv corresponds approximately to residues 98-150 and region viii to residues 237-382. The hydrophobicity plot appears remarkably similar to that of connexin32 (Fig. 4 a).

#### Comparison of Connexin43 and Connexin32

The predicted amino acid sequence of connexin43 shows areas of homology to the predicted sequence (32) of connexin32 (Fig. 5). Of residues 1-105 in connexin43 (regions i-iii), 61 (58%) are identical to their counterparts in residues 1-104 in connexin32 (regions I-III). Of residues 142-246 in connexin43 (regions v-vii), 44 (42%) are identical to their counterparts in residues 122-227 of connexin 32 (regions V-VII). Optimal alignment of the two proteins also demonstrates that many of the amino acid substitutions between the two junction molecules are conservative, suggesting that the structures of these six regions may be very similar. There is much less homology in other portions of the two molecules. Connexin43 has 20 more amino acids in region iv than connexin32 has in region IV. The COOH-terminal region of connexin43 (region viii) is substantially longer than its counterpart in connexin32; amino acid matches occur only slightly more frequently than would be predicted by chance. The nucleotide sequences for these unique areas, as well as the 3' untranslated tails, show no homology.

No regions of internal homology within connexin43 were identified. A search of the National Biomedical Research Foundation protein sequence data base and of the predicted translations of identified exons in the GenBank/Los Alamos database identified no other proteins with significant homology to connexin43. In particular, the derived sequence of the lens membrane protein, MP26, which has been suggested to be a structural component of the lens fiber junctions (16), showed no homology to either connexin43 or connexin32.

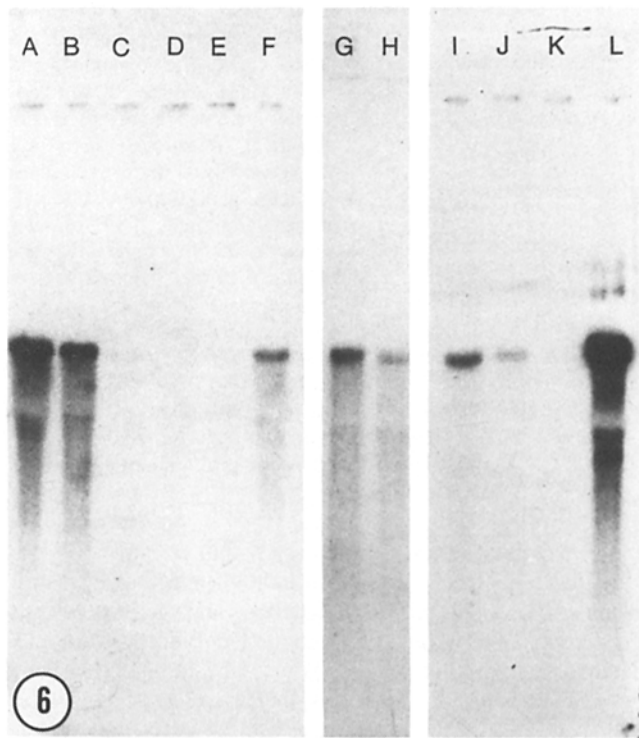
#### Northern Blot Organ Survey

Total RNAs from various rat organs were examined for homologous messages by Northern blot analysis using G2, a 1.4-kb probe that contains the entire coding region of connexin43 cDNA. Under high stringency conditions of hybridization and washing, a single band of 3.0 kb was seen in RNA from heart, term uterus, PMSG-primed ovary, and kidney. The 3.0-kb band was seen in intact and trypsinized rat lenses, but not in decapsulated lenses, suggesting that it derived from lens epithelial cells but not lens fibers (Fig. 6). No bands were seen in RNA from brain, stomach, spleen, or liver. Further high stringency Northern blots were connexin32 cDNA confirmed the previous finding (32) that a homologous 1.6-kb mRNA is expressed in liver, brain, stomach, and kidney (data not shown). The 1.6-kb band was also seen in whole lenses; however, digestion of the lenses with trypsin (12) abolished this signal, indicating that it derived from adherent ciliary epithelium. We did not find any of the 1.6-kb connexin32 message on Northern blots of RNA from ovary, uterus, spleen, or heart.

#### Discussion

This paper describes the molecular cloning of cDNA for

Figure 4. Hydropathicity plots of connexin32 (a) and connexin43 (b). Hydropathicity values (determined with a window of 20 residues) have been plotted with respect to position along the derived amino acid sequences of the connexins. Amino acid numbers are shown at the left of each plot. The plots of connexin32 and connexin43 appear remarkably similar. Each has four prominent hydrophobic regions marked with brackets (I, III, IV, VII and i, iii, v, vii), which are interspersed with more hydrophilic areas. Hydrophilic regions iv and viii are substantially longer in the heart protein than their counterparts in the liver. Approximate amino acid residues corresponding to these regions may be assigned. Connexin32 regions represent: I, 1-42; II, 43-67; III, 68-96; IV, 97-130; V, 131-166; VI, 167-188; VII, 189-216; VIII, 217-283. Connexin43 regions correspond to: i, 1-43; ii, 44-68; iii, 69-97; iv, 98-150; v, 151-187; vi, 188-207; vii, 208-236; viii, 237-382.



**Figure 6.** Hybridization of connexin43 cDNA to total RNAs from various tissues. Blots were probed at high stringency with cDNA clone G2, which contains the entire coding region. Three different blots are shown, each with a lane of heart RNA (lanes A, G, and L) for comparison. In the first blot, lanes A–F, a positive band comigrating with the 3.0-kb heart signal is seen in PMSG-stimulated ovary (B), and kidney (F). At the level of sensitivity of this experiment, no signals are evident in RNA fractions from liver (C), stomach (D), or brain (E). In this blot, 10  $\mu$ g of RNA were loaded per lane. In lanes G and H, also loaded with 10  $\mu$ g RNA, heart (G) and term uterus (H) both show co-migrating 3.0-kb bands. In the third blot (lanes I–L), RNA from heart (L) and whole lens (I) were loaded at 7.5  $\mu$ g per lane, and show the 3.0-kb signal. Trypsin-treated (J) and decapsulated (K) lens RNA fractions were loaded at 3.0  $\mu$ g per lane. The RNA from the trypsin-treated lens contains the 3.0-kb band (J), which is not detectable in the RNA prepared from the decapsulated lens (K).

connexin43. Connexin43 mRNA is abundant in myocardium, and its predicted amino acid sequence is highly homologous to connexin32, a gap junction protein abundant in liver. Evidence that this cDNA codes for a heart gap junction protein comes from the excellent agreement of the predicted sequence with the partial amino acid sequence observed in protein from isolated heart gap junctions. However, this does not yet prove that connexin43 is indeed a protein forming intercellular channels in cardiac gap junctions.

#### **Confirming that a cDNA Codes for a Gap Junction Protein**

To demonstrate that polypeptides predicted by cDNAs are gap junctional channel proteins, rigorous morphological and functional criteria need to be satisfied. Morphological evidence may be provided by EM immunocytochemical staining of the structure with antibodies either to bacterially expressed fusion protein (32) or to synthetic oligopeptides cor-

responding to the predicted sequence. Functional evidence may be provided by demonstrating that the protein can facilitate communication between cells.

Three approaches have been used to demonstrate that a protein is capable of forming a gap junction channel. In the first, intercellular communication via gap junctions, as assayed by dye and electrical coupling, is shown to be blocked by intracellular application of an antiserum specific for the putative channel-forming polypeptide (14, 22, 38, 39). One difficulty in this approach is the design of appropriate controls. Cross-linking of nonjunctional membrane proteins by an antibody may trigger a nonspecific intracellular response, resulting secondarily in closure of gap junctional channels. In addition, polyclonal antisera, even if affinity purified, may contain activity against unknown epitopes, or have a toxic activity apparently unrelated to binding of the specific protein.

In the second approach, channels are reconstituted in artificial lipid bilayers (18, 33, 44). A problem with this approach is that gap junctions are double-membrane structures while reconstituted lipid bilayers are single membranes, although an innovative double-membrane reconstitution system is currently being developed (5). Comparison of the reconstituted channel with an *in vivo* channel is difficult because the properties of gap junction channels in single membranes have not been determined *in vivo*. In addition, proteins must be very carefully purified so that reconstitution of unwanted channels does not occur. Reconstitution of proteins produced *in vitro* from cloned cDNAs could alleviate this difficulty.

In the third approach, mRNA coding for the putative junctional polypeptide may be introduced into cells and then the cells assayed for the ability to communicate (7). This type of experiment should include a demonstration that the protein coded by the foreign mRNA is being synthesized and assembled into gap junctions. The foreign channels must also be discriminated from endogenous channels, which requires that the properties of each type be measurably different.

Confirmation that a particular protein is capable of forming a gap junctional channel requires rigorous application of one or more of these methods, with close attention to the shortcomings peculiar to each. As detailed above, many of these structural and functional criteria have been satisfied for connexin32. A similar demonstration for connexin43 will require additional experimentation.

#### **Connexin mRNAs Are Not Confined to Single Organs**

The mRNAs for the two connexin molecules are found in different abundances in different organs. Connexin32 mRNA appears more abundant in liver, stomach, and brain, while connexin43 mRNA is more abundant in heart, term uterus, PMSG-primed ovary, and lens. There is a strong signal for both connexin messages in the kidney, although it is unknown whether they derive from the same or different cells. Experiments suggest that in the lens the connexin43 mRNA is localized to the epithelial cells. Crudely dissected and enzymatically digested lenses show a band by Northern analysis using the connexin43 cDNA (see Fig. 6). This signal is lost when the mRNA is prepared from decapsulated lenses, a procedure which is known to remove the bulk of the lens epithelial cells.

These comparative Northern analyses have limitations. First, while the Northern blots were performed under highly

stringent conditions, and show similarly sized mRNAs, this does not guarantee absolute mRNA identity. It is possible that identically appearing mRNAs might specify proteins with extensive sequence homology that might contain minor but key differences. Second, except for the case of the lens, RNA was prepared from whole organs, containing many diverse tissues and cell types. Thus, there is no information about which cell type expresses a given message. Third, presence of message does not guarantee that protein is being translated. Finally, the inability to detect a signal on a Northern blot does not mean that a homologous mRNA is absent, only that its abundance is too low to detect. Our results show that there is a predominance of different connexin mRNAs in certain organs, but do not rule out the simultaneous expression of both connexin messages, as clearly seen in the kidney.

### Connexin Membrane Topology

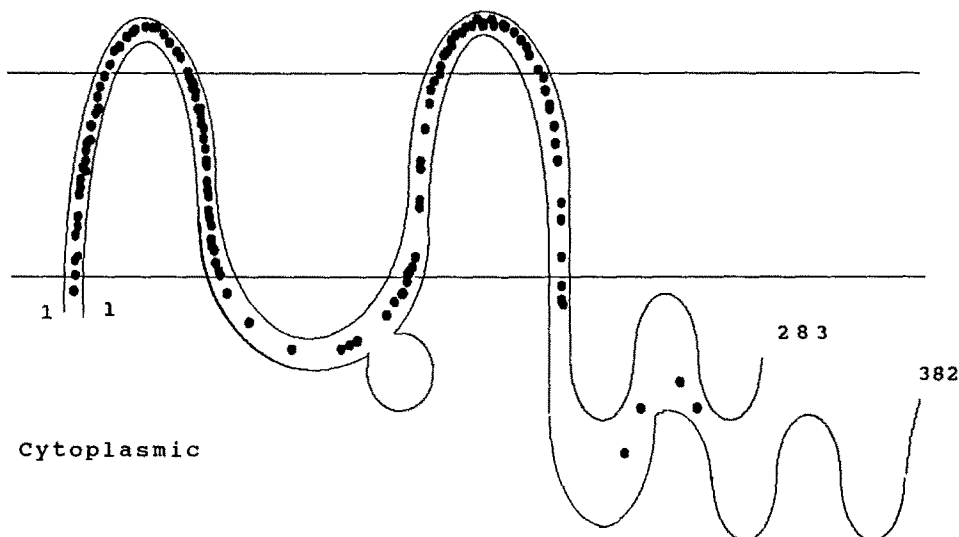
Recent studies of proteolytically treated liver gap junctions (46) have demonstrated that the COOH-terminal portion of connexin32 (region VIII) and a Lys-X proteolytic site (region IV) are located on the cytoplasmic sides of the junctional membranes. These data place both regions IV and VIII at the cytoplasmic surfaces, as drawn by Zimmer et al. (Fig. 14 in reference 46) and here in Fig. 7. The cytoplasmic localization of these regions and the assumption that the hydrophobic portions represent membrane-spanning regions suggest a topological model of the liver and heart gap junction proteins with relation to the junctional membrane (Fig. 7). In this drawing, connexin32 and connexin43 are depicted as parallel lines; the dark dots between them indicate positions of identical amino acids. The putative membrane spanning and extracellular regions of the two connexins are conserved structures, and the cytoplasmic portions of the molecules are

divergent. Each of the short extracellular loops (regions II and VI) contains three cysteine residues that are conserved between the connexin sequences. Neither the single consensus glycosylation site near the beginning of connexin32 nor any of the sites in connexin43 lie in the predicted extracellular regions. In this context, it is pertinent that no glycosylation of connexin32 has been reported.

The conserved transmembrane and extracellular structures in connexin molecules suggest that the structure of the transmembrane portion of the channels and the mechanisms of cell-cell interaction may be similar in different tissues. Previous authors have demonstrated that heterologous cells can form low resistance communication channels in culture (13). An interesting question in this regard is whether or not heterologous cells actually make heteromolecular junctions in tissue culture, or whether they express different junctional phenotypes in culture. Flagg-Newton and Loewenstein (13) have demonstrated an asymmetric physiology of heterologous cell junctions in culture, consistent with the existence of heteromolecular structures.

The unique cytoplasmic primary structures of the connexins may confer different physiological behavior. These unique regions are exposed to intracellular mechanisms of phosphorylation (2, 4, 35, 40), calmodulin binding (46), and proteolysis concomitant with protein turnover (10, 43). Physiological studies on pH sensitivity have shown that liver and heart channels have measurably different pKs of 6.4 and 6.8, respectively (34, 37, 41), properties which presumably reside at the junctional cytoplasmic surfaces. The heart junctions are obligatory conductors of electrical excitation between the myocytes, a role not shared with the liver, but possibly shared with uterine myometrium. Whether or not this functional difference can be localized to specific protein structural domains has yet to be determined.

Extracellular



Cytoplasmic

Figure 7. A model depicting topology of connexin proteins in relation to the junctional membrane, following the orientation presented by Zimmer et al. (1987). Connexin32 (1-283) and connexin43 (1-382) are drawn as parallel lines to show their optimal alignments, except in the middle of the molecules and at the COOH terminus where connexin43 has additional mass. Heavy dots are drawn between the lines at positions of identical amino acids. This model was constructed based on previous evidence that the COOH terminus and region IV of connexin32 are on the cytoplasmic side of the membrane and the assumption that hydrophobic regions span the membrane. The model reveals that the putative transmembrane and extracellular regions of the connexins are conserved and that the cytoplasmic portions are divergent.

## The Connexin Family of Proteins

We propose a new system of nomenclature, using the term connexin to identify the members of a family of proteins that are related by a high degree of conservation in their predicted amino acid sequences, prototypically shown here between connexin32 and -43. We will not attempt to further define the degree of conservation required, since only two members of the family have been characterized. We think that the connexin family of proteins will be shown to be gap junction proteins. Additional support that connexin43 is a gap junction protein comes from a strong homology with the NH<sub>2</sub>-terminal of a previously identified heart gap junction protein. However, by the criteria suggested above, connexin43 has not yet been shown to be a gap junction protein.

The family of connexins may contain additional proteins beyond the two reported here. Initial data have been presented (29) demonstrating the presence in rodent livers of an M<sub>r</sub> 21,000 protein, present in junctional plaques, which is 45% homologous to connexin32 in the NH<sub>2</sub>-terminal 20 amino acids. In addition, using the same strategy followed in this paper, we have isolated an additional homologous cDNA from rat lens fibers (Paul, D. L., E. C. Beyer, and D. A. Goodenough, manuscript in preparation). All gap junction proteins will not necessarily be members of the connexin family. Thus, the lens fiber protein, MP26, for which the entire sequence has been determined (16), is clearly not a member of the connexin family on the basis of its very different amino acid sequence.

We have proposed using the predicted polypeptide molecular mass in kilodaltons to distinguish between different members of the connexin family (e.g. connexin32, connexin43). In the event that different connexins are identified in the same organism with nearly the same mass, it may be necessary to distinguish them by using the first decimal place. As we have shown, it is likely that the same connexin mRNA is expressed in many different organs; therefore, we believe that any reference to organ (as liver, heart, or uterus) is inappropriate. Connexins have been identified from other species (23); they must be distinguished by specification of the organism.

Although there are potentially many biological roles for intercellular communication, currently only a few functions are understood. For this reason, we have no explanation for connexin diversity.

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