

Sequence and Tissue Distribution of a Second Protein of Hepatic Gap Junctions, Cx26, As Deduced from its cDNA

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Abstract. While a number of different gap junction proteins have now been identified, hepatic gap junctions are unique in being the first demonstrated case where two homologous, but distinct, proteins (28,000 and 21,000 M_r) are found within a single gap junctional plaque (Nicholson, B. J., R. Dermietzel, D. Teplow, O. Traub, K. Willecke, and J.-P. Revel. 1987. *Nature [Lond.]* 329:732-734). The cDNA for the major 28,000- M_r component has been cloned (Paul, D. L. 1986. *J. Cell Biol.* 103:123-134) (Kumar, N. M., and N. B. Gilula. 1986. *J. Cell Biol.* 103:767-776) and, based on its deduced formula weight of 32,007, has been designated connexin 32 (or Cx32 as used here). We now report the selection and characterization of clones for the second 21,000- M_r protein using an oligonucleotide derived from the amino-terminal protein sequence. Together the cDNAs represent 2.4 kb of the single 2.5-kb message detected in Northern blots. An open reading frame of 678 bp coding for a protein with a calculated molecular mass of 26,453 D was

identified. Overall sequence homology with Cx32 and Cx43 (64 and 51% amino acid identities, respectively) and a similar predicted tertiary structure confirm that this protein forms part of the connexin family and is consequently referred to as Cx26. Consistent with observations on Cx43 (Beyer, E. C., D. L. Paul, and D. A. Goodenough. 1987. *J. Cell Biol.* 105:2621-2629) the most marked divergence between Cx26 and other members of the family lies in the sequence of the cytoplasmic domains. The Cx26 gene is present as a single copy per haploid genome in rat and, based on Southern blots, appears to contain at least one intron outside the open reading frame. Northern blots indicate that Cx32 and Cx26 are typically coexpressed, messages for both having been identified in liver, kidney, intestine, lung, spleen, stomach, testes, and brain, but not heart and adult skeletal muscle. This raises the interesting prospect of having differential modes of regulating intercellular channels within a given tissue and, at least in the case of liver, a given cell.

GAP junctions are specialized regions of the plasma membrane comprised of closely packed aggregates of channels that pass small molecules between cells in contact (Bennett and Goodenough, 1978; Hertzberg et al., 1981; Lowenstein, 1979, 1981). It has been suggested, from a variety of systems, that gap junctionally mediated communication plays a central role in regulating development (for review see Caveney, 1985; see also Fraser et al., 1987), cell growth (for review see Lowenstein, 1979; see also Mehta et al., 1986), and metabolism (Sheridan et al., 1979; Sheridan and Atkinson, 1985). Modulators of junctional coupling include Ca^{++} , H^+ , voltage, and cAMP (for review see Peraccia, 1980; Spray and Bennett, 1985; see also Saez et al., 1986; Traub et al., 1987).

Gap junctions have been isolated and characterized from liver (Henderson et al., 1979; Hertzberg and Gilula, 1979; Nicholson et al., 1987), heart (Gros et al., 1983; Manjunath et al., 1985), and lens (Goodenough, 1979; Kistler et al., 1988). Biochemical analyses have defined a major (28,000- M_r) and minor (21,000- M_r) protein component of liver gap

junctions and single protein components in both heart (47,000 M_r), and, apparently, lens (70,000 M_r), although, in the latter case, the role of the unrelated but more abundant 26,000- M_r protein (MIP 26) remains an issue of contention (Paul and Goodenough, 1983; Bok et al., 1982; Gorin et al., 1984). Isolation of their corresponding cDNAs has established a clearer view of this family of proteins (as initially described in Gros et al., 1983; Nicholson et al., 1985, 1987; Kistler et al., 1988) now referred to as connexins (Beyer et al., 1987). Based on their calculated formula weights, the nomenclature describing the known mammalian gap junctional proteins has now been modified so that (a) the liver 28,000- M_r protein is referred to as connexin 32 (or Cx32) (Paul, 1986); (b) the liver 21,000- M_r protein is referred to as Cx26 (this manuscript); (c) the heart 47,000- M_r protein is referred to as Cx43 (Beyer et al., 1987); and (d) the lens 70,000- M_r protein is tentatively referred to as Cx46 (Beyer et al., 1988).

Beyond the obvious variation in size among these junctional proteins, a 40-60% divergence in their primary sequences has also been established, both by direct amino-terminal sequencing of Cx32, Cx26, Cx43, and the 70,000-

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M_r protein of eye lens (Nicholson et al., 1985, 1987; Kistler et al., 1988) and comparisons of the complete sequences deduced from the cDNAs of Cx32 (Paul, 1986; Kumar and Gilula, 1986), Cx43 (Beyer et al., 1987), and Cx26 (here). In contrast, a given connexin is relatively well conserved between species as evidenced by a direct comparison of sequences (Kumar and Gilula, 1986; Gimlich et al., 1988) and the surprising immunological cross-reactivity of mammalian and coelenterate gap junctions (Fraser et al., 1987).

Some other proteins, unrelated to the connexins, have also been associated both morphologically and immunologically with gap junction-like structures, notably MIP 26 in lens (see above) and relatively abundant 16,000- M_r (in mammals; Finbow et al., 1983) and 18,000- M_r (in arthropods; Finbow et al., 1984; Berdan and Gilula, 1988) proteins in a number of tissues. Several lines of evidence suggest that MIP 26 may form channels (Zampighi et al., 1985; Johnson et al., 1988), but their gap junctional nature remains an open question.

The variations in protein content of gap junctions occurring from tissue to tissue has generally been presumed to reflect tissue- or cell type-specific requirements for electrical coupling, metabolic cooperation, or signal transmission. An even broader role for junctional polymorphism was recently suggested by the demonstration of two related but distinct junctional proteins (Cx32 and Cx26), not only in the same cell, but in the same junctional plaques (Nicholson et al., 1987). This either (a) reflects a requirement for two routes of intercellular coupling between hepatocytes, presumably distinguished by channel properties and/or regulatory phenomena, or (b) indicates that, contrary to current models, gap junctional channels can be heteropolymeric, allowing for the possibility of subtle variation in channel properties by variation in the stoichiometry of the subunits, as seen in different species (Henderson et al., 1979; Nicholson et al., 1981) and different domains of the liver (Traub et al., 1989). We report here the isolation of cDNA clones for Cx26. Cx26 is shown to have the characteristics of an integral membrane, and probably channel-forming, protein. Comparisons of deduced amino acid sequences of Cx26 and Cx32 (and recently Cx43 from heart; Beyer et al., 1987) reveal patterns of conserved and variable domains suggestive of differences in the regulatory sites of each of these channel proteins.

Materials and Methods

Screening of cDNA Library

Recombinant phage were isolated from a rat liver cDNA library in λ gt11 (Mueckler and Pitot, 1985; 30,000 plaques per plate) and then pretreated as described by Maniatis et al. (1982). Filters were prehybridized at 50°C for 6 h in 6 \times SSC (20 \times SSC: 3 M NaCl, 0.3 M Na citrate), 5 \times Denhardt's solution (100 \times Denhardt's solution: 2% [wt/vol] ficoll, 2% [wt/vol] BSA, 2% [wt/vol] polyvinylpyrrolidone), 10% dextran sulfate, 1% (wt/vol) SDS, 0.1% Na pyrophosphate, and 100 μ g/ml heat-denatured salmon sperm DNA. Hybridization was carried out after the addition of the 5'-labeled oligonucleotide probe (5 \times 10⁵ cpm/ml, 3.5 \times 10⁸ cpm/ μ g) at 50°C for 24 h. The filters were then washed four times in 6 \times SSC, 0.1% sodium pyrophosphate for 20–30 min at room temperature and exposed to XAR-5 film (Eastman Kodak Co., Rochester, NY) at –70°C.

Plaques that were positive on duplicate replicas were picked and purified by two successive platings and rescreenings before isolation of the phage DNA. Phage DNA was isolated according to Davis et al. (1986) using a plate rather than a liquid culture lysate. The cDNA insert was excised by Eco RI and purified by electrophoresis in low-melting point agarose (FMC

Bio Products, Rockland, ME). Isolation of the longer Cx26 clone was achieved by rescreening \sim 10⁶ phage from the original library at high stringency (Maniatis et al., 1982) with a nick-translated probe of the initial Cx26 cDNA insert (2 \times 10⁵ cpm/ml, 2 \times 10⁷ cpm/ μ g).

Northern Blot Analysis

Total RNAs from heart, intestine, liver, lung, kidney, spleen, stomach, testis, skeletal muscle, and mouse liver were isolated by homogenization in guanidine thiocyanate followed by a CsCl gradient in which the RNA is banded rather than pelleted (Chirgwin et al., 1979; Fyrberg et al., 1980). Salts were then removed by successive ethanol precipitations. A₂₆₀/A₂₈₀ for this RNA was typically 1.8–2. 20 μ g of total RNA from each tissue was glyoxylated and electrophoresed in a 1% agarose gel according to Maniatis et al. (1982). The gels were capillary blotted in 10 \times SSC onto Gene Screen Plus membrane (DuPont Co., Wilmington, DE). The RNA blots were prehybridized in 50% formamide, 6 \times SSC, 1% SDS, 10% dextran sulfate, and 100 μ g/ml heat-denatured salmon sperm DNA for 6 h at 42°C and then hybridized in the same buffer for 24 h at 42°C after the addition of the denatured, nick-translated probe (4 \times 10⁵ cpm/ml). The probes used were (a) a full-length (1.6-kb) cDNA insert of Cx32 (provided by David Paul [Harvard Medical School, Cambridge, MA]); and (b) a Hinc II–Bst XI fragment of Cx26 cDNA (representing 72% of the coding region and no untranslated regions). Washes after hybridization were done twice for 5 min with 2 \times SSC at room temperature, twice for 30 min with 2 \times SSC, 1% SDS at 60°C, and twice for 30 min with 0.1 \times SSC at room temperature. The membranes were then exposed to XAR-5 film (Eastman Kodak Co.) at –70°C.

Quantitation of message levels for Cx32 and Cx26 in mouse and rat liver used similar autoradiograms to those in Fig. 7 (see Nicholson and Zhang, 1988). Equivalent levels of total RNA, as determined by A₂₆₀ were loaded on the original glyoxal gel, and ethidium bromide staining was used after blotting to ensure efficient transfer of RNA. Nick-translated probes of identical specific activity were produced for both Cx32 and Cx26. After hybridization and washing, multiple exposures of the Northern blot to x-ray film were made so that all measurements could be made in the linear response range of the film. Quantitation was achieved by scanning laser densitometer (LKB Instruments, Inc., Bromma, Sweden) interfaced with an Apple IIc-based integrator (Apple Computer Corp., Cupertino, CA). Correction was made for the length and base composition of each probe and the exposure time of the autoradiogram.

Southern Blot Analysis

Large molecular weight DNA was isolated from rat liver according to Maniatis et al. (1982) and digested by different restriction enzymes. About 10 μ g of DNA fragments were separated on a 1% agarose gel and capillary transferred to Gene Screen Plus membrane (DuPont Co.). Prehybridization, hybridization with various restriction fragments of the Cx26 cDNA (³²P-labeled by nick translation), and washing were all done as for the Northern blots.

DNA Sequencing

The Cx26 cDNA insert was excised from phage DNA with Eco RI and subsequently separated by, and purified from, a low melting point agarose gel (Perbal, 1984). The purified DNA inserts were then subcloned into pGEM-Blue vectors (Promega Biotec, Madison, WI) using the protocol suggested by the supplier. The recombinant DNA was transformed into *Escherichia coli* JM 109. Plasmid DNA was purified as suggested by the supplier, and different restriction fragments were produced to give a series of overlapping sequences which were subcloned into the same vector. The sequencing reactions used the Klenow fragment of DNA Pol I in the presence of [α -³⁵S]dATP (New England Nuclear, Boston, MA) using the dideoxy chain termination method (Sanger et al., 1977). For clarification of particularly intractable regions, reverse transcriptase was used in the sequencing reactions.

Oligonucleotide Synthesis and Labeling

A 48-base oligonucleotide mixture was synthesized on a DNA synthesizer (Model 381A; Applied Biosystems, Inc., Foster City, CA) using phosphoramidite chemistry. Full-length product was separated on a 20% polyacrylamide gel, visualized by UV shadowing, excised and eluted by diffusion. Purified oligonucleotide was labeled using [γ -³²P]ATP (ICN Radiochemicals, Irvine, CA) and T4 polynucleotide kinase (Bethesda Re-

search Laboratories, Gaithersburg, MD). The reaction was done in 50 mM Tris, pH 7.6, 10 mM MgCl₂, 5 mM DTT, 0.1 mM Spermidine, and 0.1 mM EDTA at 37°C for 1.5 h. The labeled oligonucleotide was separated from free nucleotides on a 15% polyacrylamide gel and eluted overnight by diffusion from the gel. Typical specific activities were 3.5×10^8 cpm/ μ g.

Preparation and Affinity Purification of Anti-Peptide Antibodies

A Cx26-specific peptide (amino acid residues 101–119) was prepared on an automated peptide synthesizer (Model 9500; Bioscience, San Rafael, CA) using standard Merrifield chemistry and hydrogen fluoride cleavage and deprotection. Purification of the peptides by HPLC used a 0–60% acetonitrile gradient in 0.1% trifluoroacetic acid on a C-18 column.

Rabbits were immunized with 1 mg of peptide in complete Freund's adjuvant (mixed 2:1 with aqueous peptide solution) intradermally and intramuscularly. The animals were boosted 2 mo later with 0.5 mg peptide in complete Freund's adjuvant and bled 1 wk after boost. For purposes of affinity purification, HPLC-purified peptide was conjugated via Schiff's base to the free aldehyde groups on the membrane filter of a MAC-25 cartridge (Membretec) in accordance with the manufacturer's instructions. 700 μ l of serum was passed through the filter which was then washed with PBS to remove unbound proteins. The specific antibodies were then eluted with 0.1 M glycine, pH 2.2, and immediately equilibrated with PBS, 0.01% sodium azide by passage over a Sephadex G-50–spun column.

Immunoblot and Electron Microscopic Immunolabeling

Gap junctions were isolated using a modified procedure of Hertzberg (1984) in which the initial gradient for isolation of plasma membranes was deleted. About 0.2 μ g of protein from isolated gap junctions was separated on a 15% polyacrylamide gel and transferred onto a polyvinylidene difluoride membrane (Millipore Continental Water Systems, Bedford, MA). The blots were blocked for 1.5 h at 37°C in TBS (0.01 M Tris, pH 7.4, 0.9% NaCl) containing 3% BSA. Reactions with primary antibody at a dilution of 1:100 in TBS with 0.2% BSA was done at 37°C for 2 h. After three washes in TBS containing 0.2% BSA, the blots were treated with 2 μ g/ml alkaline phosphatase-conjugated protein A (Cappel Laboratories, Malvern, PA) for 1 h at room temperature. Color development was achieved with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate *p*-toluidine according to the vendor's (Bethesda Research Laboratories) instructions.

For electron microscopic immunolabeling, 10 μ l of isolated mouse liver gap junctions (0.01 μ g/ μ l of protein) was laid on a copper grid and air dried. The grids were then blocked with 0.5% BSA in PBS at 37°C for 30 min. After three washes for 5 min, the grids were incubated for 1 h at 37°C in affinity-purified preimmune or immune serum (1:5 dilution) in PBS containing 0.2% BSA and then washed three times for 5 min before incubating with goat anti-rabbit IgG conjugated to 10-nm gold particles for 30 min at room temperature. The grids were washed five times in PBS containing 0.2% BSA, pH 7.2, and twice in water and then stained by two 1-min incubations with 2% phosphotungstic acid in water at pH 7.2.

In Vitro Transcription and Translation

About 3 μ g of recombinant DNA linearized with Hind III was transcribed in a 100 μ l reaction mixture (using a kit from Promega Biotec) in the presence of five A₂₆₀ U/ml of cap analogue (m⁷G[5']ppp[5']G). RNA transcripts were purified by treatment with RQ1 DNase, phenol/chloroform and chloroform extraction, and ethanol precipitation according to the protocols provided by Promega Biotec. RNA from each reaction was dissolved in 14 μ l diethylpyrocarbonate-treated H₂O containing 1 U/ μ l RNasin (Promega Biotec). The yield of RNA was typically 5 μ g per reaction. Translations in 50 μ l of rabbit reticulocyte lysate, using a kit from Promega Biotec, were allowed to proceed at 30°C for 90 min with 14 ng/ μ l RNA and 1 μ Ci/ μ l [³⁵S]methionine. Translation products were analyzed by 12.5% PAGE.

Results

Identification and Isolation of cDNA Clones

An oligonucleotide mixture (Fig. 1) was synthesized to represent the antisense DNA corresponding to the amino-

terminal 16 residues of Cx26 (Nicholson et al., 1987). In designing the oligonucleotide probe, mammalian codon use, GT base pairing (Lathé, 1985), and the ability of deoxyinosine to base pair with all nucleotides other than C (Ohtsuka et al., 1985) were used to synthesize a 64-fold degenerate 48-mer. From 300,000 plaques of a λ gt11 rat liver cDNA library, screened with the kinased oligonucleotide, one positive clone containing a 1.1-kb insert (termed Cx26-1) was found on duplicate filters. Rescreening of 10⁶ plaques from the same library with this cDNA at high stringency (6 \times SSC, 50% formamide; 42°C) revealed three positive clones of which two appeared identical to Cx26-1. The other, designated Cx26-2, contained a 2-kb insert that overlapped at its 5' end with Cx26-1 but contained an additional 1.3 kb of 3' sequence. Splicing of these cDNAs at their common restriction site yield a fused construct (Cx26-F) of \sim 2.4 kb. This apparently represents the majority of the original mRNA since the message recognized by these cDNAs in rat and mouse liver has an estimated size of 2.5 kb (Nicholson and Zhang, 1988; see also Fig. 7).

The Isolated cDNAs Code for a Member of the Connexin Family of 26,453 mol wt

Sequencing of Cx26-1 and Cx26-2 (sequence and restriction map are shown in Fig. 2) revealed a single long open reading frame of 678 nucleotides, beginning at the first ATG codon encountered in Cx26-1 (278 nucleotides from the 5' end). This has the appropriate context for a eukaryotic translational initiation site with an A three nucleotides upstream and a G immediately downstream (Kozak, 1984, 1986). The protein encoded by this open reading frame has a molecular weight of 26,453 and an amino-terminal sequence identical to that determined directly from the 21,000-*M_r* protein in isolated rat and mouse liver gap junction fractions (Nicholson et al., 1987). The sequence also shows extensive homology throughout its length with two previously cloned junctional proteins (Cx32 and Cx43). Alignment of the coding region of Cx26 with Cx32 (Paul, 1986) requires the insertion of a gap of three bases (corresponding to amino acid 104 of Cx26) in the Cx32 sequence (Fig. 3). Consistent with this, and the original Cx32-to-Cx43 comparison (Beyer et al., 1987), a gap of 57 bases (19 amino acids) must be introduced into Cx26 at approximately the same location to maintain alignment with Cx43. It should be stressed, however, that the minimal similarity in the Cx43, Cx32, and Cx26 sequences in this region makes the exact location of the gap a rather arbitrary assignment.

The overall nucleotide homology between Cx32 and Cx26 is 66% within the open reading frame, but this drops to only 20–30% immediately upstream and downstream of the proposed initiation and termination sites, respectively. It therefore seems unlikely that the coding region for Cx26 used in vivo would extend beyond the TAA at position 678. This conclusion is supported by the presence of multiple stop codons in all three reading frames immediately after the end of the open reading frame. Within the open reading frame, homology with the other connexins is not uniform. Three regions (designated as domains A [amino acids 1–23], C [amino acids 97–131], and E [amino acids 217–226] in Fig. 3) share nucleotide homologies of 39–57% with Cx32 as compared with 72–92% for the intervening domains (coding amino

	1				5					10					15		
a.a. Sequence	H ₂ N —	Met Trp	• Asp	• Trp	• Gly	• Thr	• Leu	• Gln	• Ser	• Ile	• Leu	• Gly	• Gly	• Val	• Asn	• Lys	• His
Codons	5'—	ATG TGG	GAY	TGG	GGN	ACN	CTN TTU	CAU	TCN AGY	AT ^Y A	CTN TTU	GGN	GGN	GTN	AA ^Y	AAU	CAY —3'
48 mer	3'—	ACC	CTI	ACC	CCI	TGI	GA ^C G	GT ^C T	I ^G C	TAI	GA ^C G	CCI	CCI	CA ^C I	TTI	TT ^C T	GTG —5'

Figure 1. Amino acid sequence and derived DNA sequence for synthetic oligonucleotide mixture. The amino acid sequence of the 21,000-*M_r* gap junction protein (Cx26) is from Nicholson et al. (1987). The corresponding codons for each amino acid (sense DNA) are shown on the second line. The third line shows the sequence of the oligonucleotide mixture synthesized corresponding to the antisense strand. At degenerate positions, deoxyinosine was used wherever mammalian codon use tables did not suggest that G was favored. Where G was favored (codons 7, 13, and 15), C was used. To reduce the occurrence of consecutive inosine bases, only the two heavily favored codons for leucine were used for codons 6 and 10. In codon 16, only G was used as this can also form stable G-T mismatches. Due to the ambiguity in the signal from protein sequencing for the amino-terminal residue, we simply chose what we felt to be the most likely (Trp), a choice we subsequently found to be wrong. Y, C and T; U, A and G; N, A, G, C, and T.

acids 24–96 and 132–216). This same pattern of conserved and variable domains is also seen in comparisons with Cx43 and other nonmammalian connexins (Cx38 and Cx30; Ebihara et al., 1989; Gimlich et al., 1988), although levels of homology tend to be reduced. The significance of these patterns of similarity and divergence will be considered later with respect to the proposed connexin structure within the gap junction.

The Translation Product of the Cx26 cDNA Is Indistinguishable from the 25,000-*M_r* Protein in Isolated Liver Gap Junctions

When added to a rabbit reticulocyte lysate containing [³⁵S]-methionine, capped SP6 RNA polymerase transcripts of the Cx26-1 cDNA were found to direct the synthesis of a major labeled product of 25,000-*M_r* on SDS-polyacrylamide gels (Fig. 4 A). Although this mobility is different from that of 21,000 *M_r*, originally reported, with our current gel system this is the identical mobility to that observed for the minor component of both rat and mouse liver gap junctions (Fig. 4 E). In most experiments (e.g., Fig. 4 A), a prominent band of 43,000 *M_r* is also evident. Given that it is immunoprecipitated, along with the 25,000-*M_r* polypeptide, by an antibody specific to Cx26 (see below), it seems likely to represent the dimeric form typically found in SDS gels of isolated junctions. Some truncated or degraded products of slightly lower molecular weight, which are similarly immunoprecipitated, are also frequently seen (smeared material below the 25,000-*M_r* band in Fig. 4 A). None of these bands are found when antisense RNA (SP6 transcript of a plasmid with the cDNA inserted in the opposite orientation) was added to the reticulocyte system.

Final confirmation of the identity of the protein encoded by the open reading frame of the Cx26 cDNA was achieved through the use of polyclonal antibodies raised to a synthetic peptide from the deduced sequence of Cx26 (specifically, amino acids 101–119). In addition to immunoprecipitation of the products of *in vitro* translation referred to above, these antibodies were also analyzed for their binding characteristics to isolated gap junctions. On Western blots of isolated mouse gap junctions, the antibody is highly specific for Cx26, showing no cross-reactivity with the major Cx32 component (compare Western blot in Fig. 4 C with Coomassie-stained gel in Fig. 4 E). Further evidence of the integral

role of the Cx26 protein in the structure of the gap junction is provided by the specific decoration of negatively stained, isolated mouse liver gap junctions with gold beads by way of the Cx26 (amino acids 101–119) antibody (Fig. 5). Other structures (e.g., membrane vesicles, cytoskeletal filaments, etc.) were not labeled when cruder junctional preparations were examined (data not shown).

Structural Features of the Cx26 Gene As Deduced from the cDNA

A preliminary analysis of the genomic structure of the Cx26 was carried out on various restriction digests of rat genomic DNA separated on Southern blots and hybridized with different nick-translated restriction fragments of the Cx26 cDNA. Four nonoverlapping probes spanning the full length of the cDNA were used (see Fig. 6). Single bands detected by probe II (Hinc II, Hind III, and Sma I digests) and probe III (Eco RI, Hinc II, and Pst I) strongly suggest that the Cx26 gene is present in single copy per haploid genome. In addition, a common ~2-kb Hinc II fragment is detected by probes II, III, and IV. Since the cDNA itself has two Hinc II sites (separating probes I and II [nucleotide 317] and within probe IV [nucleotide 2,215]) separated by ~1.9 kb, this suggests that no introns (or only a very small one) exist within this region. This includes virtually the entire coding domain and appears to be a similar situation to the Cx32 gene, which also lacks introns within the coding region (Miller et al., 1988). In contrast, probe I recognizes multiple bands in Eco RI, Hinc II, and Pst I digests that would not be predicted based on restriction sites present in the cDNA sequence. Thus, it seems likely that, as reported for Cx32, the Cx26 gene contains an intron in the 5' untranslated region. A further possible intron, this time in the 3' untranslated region, is suggested by the detection of less than or equal to two fragments by probe IV in Hind III and Xba I digests of genomic DNA. Only one would have been predicted from the cDNA. It is possible that these multiple bands could have originated from partial digests or even crosshybridization with related sequences by these particular probes. However, this multiplicity of bands was not seen in the same digests hybridized with other probes (i.e., probes II and III) (arguing against the possibility of partial digestion) nor in all digests hybridized with probes I and IV (arguing against crosshybridization as a possibility).

A

CGGGCCGTCGGCTCTCCAACTCGCAGCCAGTC -241
 GGCBCGTCCCGCTACTGAGCGCAGCCTCCACCAGGATCCGCGGGACCAGCTCGGGATCAGCCGGGACCCACTTCTGA -161
 CCAACCCAGGAGCGGCCGATACCCACTCCCGACCAACCCGCGACCGACCCAGGGACCCACTCCGGACCTGCTCCTTACA - 81
 GGGGACAGCGCCTCGCGCTTCCCGCCGCCAGCGCCCGCACGCTCCTCGGGACACAGTGCCAAACCATCCAGAGGACAAG - 1

ATG GAT TGG GGC ACA CTA CAG AGC ATC CTC GGG GGT GTC AAC AAG CAC TCC ACC AGC ATT 60
 M D W G T L Q S I L G G V N K H S T S I 20
 GGG AAA ATC TGG CTC ACT GTC CTC TTC ATC TTC CGC ATC ATG ATC CTC GTG GTG GCC GCG 120
 G K I W L T V L F I F R I M I L V V A A 40
 AAG GAG GTG TGG GGA GAT GAG CAA GCC GAT TTT GTT TGC AAC ACT CTC CAG CCT GGC TGT 180
 K E V W G D E Q A D F V C N T L Q P G C 60
 AAG AAT GTG TGC TAC GAC CAC TAC TTC CCC ATC TCT CAC ATC CGG CTC TGG GCT CTG CAG 240
 K N V C Y D H Y F P I S H I R L W A L Q 80
 CTG ATC ATG GTG TCC ACG CCG GCC CTC CTG GTA GCT ATG CAC GTG GCC TAC CGG AGA CAC 300
 L I M V S T P A L L V A M H V A Y R R H 100
 GAA AAG AAA CGG AAG TTC ATG AAG GGA GAG ATA AAG AAC GAG TTT AAG GAC ATC GAA GAG 360
 E K K R K F M K G E I K N E F K D I E E 120
 ATC AAA ACC CAG AAG GTC CGT ATC GAA GGG TCC CTG TGG TGG ACC TAC ACC ACC AGC ATC 420
 I K T Q K V R I E G S L W W T Y T T S I 140
 TTC TTC CGG GTC ATC TTC GAA GCT GTC TTC ATG TAT GTC TTT TAC ATC ATG TAC AAT GGC 480
 F F R V I F E A V F M Y V F Y I M Y N G 160
 TTC TTC ATG CAG CGT CTG GTG AAG TGT AAC GCC TGG CCT TGT CCC AAT ACA GTG GAC TGC 540
 F F M Q R L V K C N A W P C P N T V D C 180
 TTC ATT TCC AGG CCC ACA GAA AAG ACT GTC TTC ACG GTG TTC ATG ATC TCT GTG TCT GGA 600
 F I S R P T E K T V F T V F M I S V S G 200
 ATT TGC ATC CTG CTA AAC ATC ACA GAG CTG TGC TAT CTG TTC ATT AGG TAT TGC TCA GGG 660
 I C I L L N I T E L C Y L F I R Y C S G 220
 AAG TCC AAA AGA CCA GTC TAA 681
 K S K R P V - 226

TGCATTGCCTGGCTGTTAAGCAAAGATGAGGGAGAGGATGAGGCCAACCTGTGCTTAGTTATCAGAGTTCAGCTACCAGCA 761
 TCTCCCGGGCAAACATTCCACCTTAAATGCCCGCCATTGAAGTCCCGCAGGCTCCCATGAAACTCCAGAAGCCTCC 841
 ATGGGCTCCCTTCCCAAAGCTCCAAACAAGGCCCAATTCATGCTGTATTAAATGGTTCTAAAGTTAGTTAGAC 921
 CCCGTGCTGGTGTGACTATGCTTTAGGATACATTACAGTTTAAACAAGGGATCTCACATTGTTTCTCTCTCTGAGG 1001
 ACAGGAGACATGAGCCAGTCTGAGGAAGGTACAGAGAAAGTTCCTTCTCCGGGTCCCTTCCCAAGTTGCCCCAG 1081
 TTAAGGGTAAAGAATCTTCGTTCTGTTATTTCTTTTCATAGTTAAGTTGCAACAATGGACAAGAGCTATTAAATGTT 1161
 AAGCTAGCTGTGCTCTTTTTTTTTTTTTTAAATGAAAACCTTAAATGATAGGTTCTTTTGTCTTAAATGATCTGGAA 1241
 AGCATTATACATTCTCCTATTTTCAGAGGTTCCGTTTGTGATGTGAGCATGGTGATAACAGATCTCACAAGGCTTTA 1321
 AACGTTGGCCTTTTGGTTATGGGAAACCTGGGCTGTGGCTGAGAGCCACCTACTGTATTTCATCCTTAGTGTGCTGAG 1401
 TACAGCCCGCAACAACGTTACAGCCTGTCTCAAATGAGACAAACTGGAAGCTTCTCGTTAGCTTCTGACAAGAAGAGG 1481
 CCTTGATTAATAATTTCAACCGTAATTTGTGTAAGAGGAGATAGGTTATGCCTACAACGCCCCCTGCCATGAGCCTA 1561
 ACTCAGCCCCCTCCACCCCGAGCTCGTCTACTCTGTAGCTGTGGGATGTGGCAGTCAGTATCAAAGACTTCATGAGTT 1641
 TGCTTGGGAATTTCACTGCATGGTACAATTTAATGGTGCAGAAACAAGATGGGGTGGTTTTCAAAGAACCAGTAAACT 1721
 TCTAGACTCTAAATCCTGTTGATTAATAACTGAGTTTTCTACTTTGAAATGTCTGTTTGCCTCCCTTTTCAGATTGCTT 1801
 CTAACCTGGAAACAGAAATGTTGATATTTGGAAAAATAGAAGAACTAGTTTAGGTCATGTGTAACTTTCTAGGACA 1881
 AGTTGAACCTTAGCATTGTCTTCTGCTGATGTGTTGTCACAAGATGACAGTCAACAAATCCAACAGGGGACACTTCT 1961
 TCCTGCAAGAATGTCTGTTGGGAAGCCATTCTGTAACAATAAATAAGAGTTGTGGTTTAAAGTCTACACTATTTTACCTA 2041
 ATGAAGAACTATTGCTGATGTTTCAGAAATTCGACATTTGAAAGGTGTTTTGCCAATACGGG 2102

B

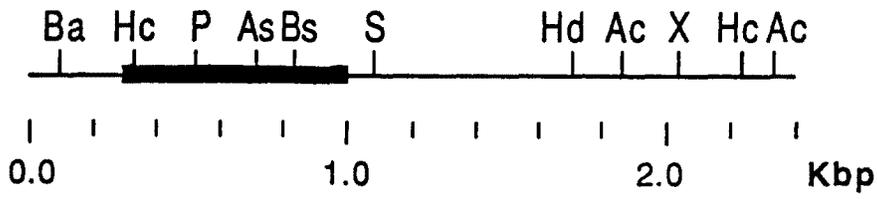


Figure 2. Nucleotide and derived amino acid sequence of Cx26-F cDNA. (A) The complete nucleotide sequence of Cx26 is shown with numbers starting from the initiator codon ATG. The derived amino acid sequence is shown in single letter code below the nucleotide sequence. The nucleotide sequence corresponding to the oligonucleotide mixture is underlined (*dashed line*). Other underlined sequences are the self complementary sequences proposed to form a loop in the 5' untranslated region (*dotted line*) and the multiple stop codons in all three reading frames in the 3' untranslated region (*solid lines*). The predicted protein has a molecular weight of 26,453. Amino acid residues (1-18) match the protein sequence data of Nicholson et al. (1987). (B) The restriction map of Cx26 is shown below the cDNA sequence. Restriction sites are those for Bam HI (*Ba*), Hinc II (*Hc*), Pst I (*P*), Asu II (*As*), Bst XI (*Bs*), Sma I (*S*), Acc I (*Ac*), Hind III (*Hd*), and Xma I (*X*).

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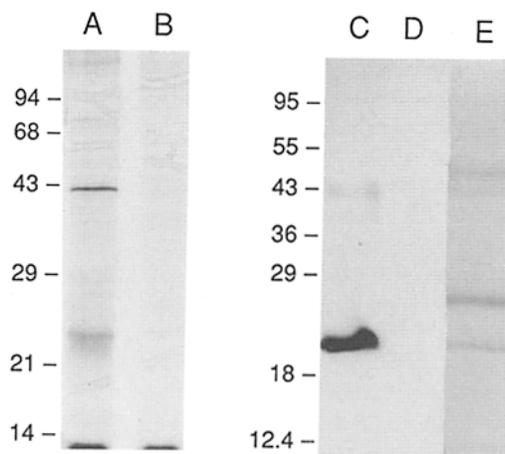


Figure 4. In vitro translation of Cx26 cDNA. Sense (lane A) or antisense (lane B) RNA transcripts of the Cx26 cDNA were translated in vitro in a rabbit reticulocyte lysate in the presence of [³⁵S]methionine. After separation on a 12.5% SDS-polyacrylamide gel, the labeled products were visualized by autoradiography. Specific products from the sense RNA have identical relative mobilities with the minor, 25,000-*M_r*, component of isolated mouse liver gap junctions (lane E) and its dimeric form (43,000 *M_r*). Approximately 0.2 μg of isolated gap junctions were separated on a 12.5% polyacrylamide gel and either stained by Coomassie blue (lane E) or blotted to polyvinylidene difluoride membranes and reacted with anti-Cx26 (101-119) affinity-purified serum (lane C) or preimmune serum (lane D). The antibody is clearly specific for the minor, 25,000-*M_r*, protein (previously called 21-kD protein; Henderson et al., 1979; Nicholson et al., 1987), at least in the denatured form. Marker proteins for each gel (mobilities marked in kilodaltons) are shown on the left.

Cx26 RNA Is Coexpressed with Cx32 RNA in a Variety of Tissues

Northern blots of rat and mouse liver RNA, when probed with the nick-translated Cx26 cDNA, showed a single band of 2.5 kb, only slightly larger than the combined lengths of our cDNAs (i.e., Cx26-F is 2.4 kb long). Based on densitometry of autoradiographs similar to those shown in Fig. 7 but using probes of identical specific activity for both Cx32 and Cx26, the Cx26 message is ~8.5-fold higher in mouse than rat (Nicholson and Zhang, 1988). This is consistent with the relative protein levels in the two tissues (Nicholson et al., 1987). The ratio of Cx26 to Cx32 message (2.5 and 1.6 kb, respectively) in rat (1:50) and mouse (1:5) liver are comparable with, but consistently lower than, the observed abundance of the respective proteins in isolated liver fractions from rat (1:20) and mouse (1:2) (Nicholson et al., 1987; Nicholson and Zhang, 1988). The possible significance of this is discussed below.

Total RNAs from rat heart, intestine, lung, kidney, spleen, stomach, testis, and skeletal muscle were also screened on Northern blots for the presence of mRNA coding for Cx26 and Cx32. Nick-translated probes from the full-length cDNA

insert of Cx32 (from Paul, 1986) and a slightly truncated coding region of Cx26 (Hinc II–Bstx I fragment) were used. As shown in Fig. 7, clear bands of ~2.5 kb for Cx26 and 1.6 kb for Cx32 were observed in rat intestine, liver, kidney, and stomach and mouse liver. Low levels of message for Cx32 were also detected in lung, spleen, and testis. No message for either connexin was detected in adult heart and skeletal muscle. This is consistent with biochemical analyses of heart gap junctions (Gros et al., 1983; Beyer et al., 1989) and the absence, morphologically, of gap junctions in skeletal muscle (Keeter et al., 1975). Together, these results show that Cx32 and Cx26 are coexpressed in a variety of, but not all, tissues. While the relative levels of Cx26 and Cx32 message clearly vary greatly (Fig. 7), no clear case of expression of one without the other was detected in this study. However, see Discussion for a consideration of the limits of this analysis. Under the high stringency conditions used, no multiple variants or close relatives of Cx32 or Cx26 were detected, except in kidney. Here, bands of ~1.5 and 0.8 kb were identified by the Cx26 probe. We do not currently know if these arise from partial but specific degradation of the 2.5 kb message or heterogeneity in the mature message size or if they derive from related connexin genes.

Discussion

In this study, we have isolated and characterized cDNAs from rat encoding the second, minor component of liver gap junctions, previously referred to as the 21,000-*M_r* protein. The combined cDNAs represent virtually all of the 2.5-kb message and contain the complete coding region for a protein with a calculated molecular weight of 26,453. The extensive homology between this protein and the previously characterized gap junctional proteins, Cx32 (66% nucleotide homology) and Cx43 (54% nucleotide homology), leads us to designate it as Cx26. Several lines of evidence confirm Cx26's identity as the 21,000-*M_r* protein of isolated liver gap junctions. Not only does the amino-terminal sequence deduced from the clone match that determined directly from the 21,000-*M_r* protein (Nicholson et al., 1987), but antibodies raised to portions of the deduced sequence also bind specifically to isolated gap junctional structures (Fig. 5). In addition, RNA transcripts from the cDNA were shown to direct the synthesis of a polypeptide of identical mobility to the minor protein component of isolated hepatic gap junctions (Fig. 4).

The Cx26 protein displays the same characteristics common to other members of the connexin family. Comparison of the deduced and directly determined amino acid sequences demonstrates the lack of a cleavable signal sequence, an observation consistent with previous studies of Cx32 (Nicholson et al., 1981; Paul, 1986; Kumar and Gilula, 1986) and Cx43 (Nicholson et al., 1985; Beyer et al., 1988). Hydropathy plots (Kyte and Doolittle, 1982) indicate the presence of four 20–24-residue transmembrane spans interspersed by reverse turns predicted by the paradigms of Chou

sequence is divided into nine domains (1–4 are hydrophobic, putative transmembrane domains; A, C, and E are proposed to be cytoplasmic domains; B and D are proposed to be extracellular domains). It is readily evident that the proposed cytoplasmic domains (A, C, and E) show little conservation between the proteins.

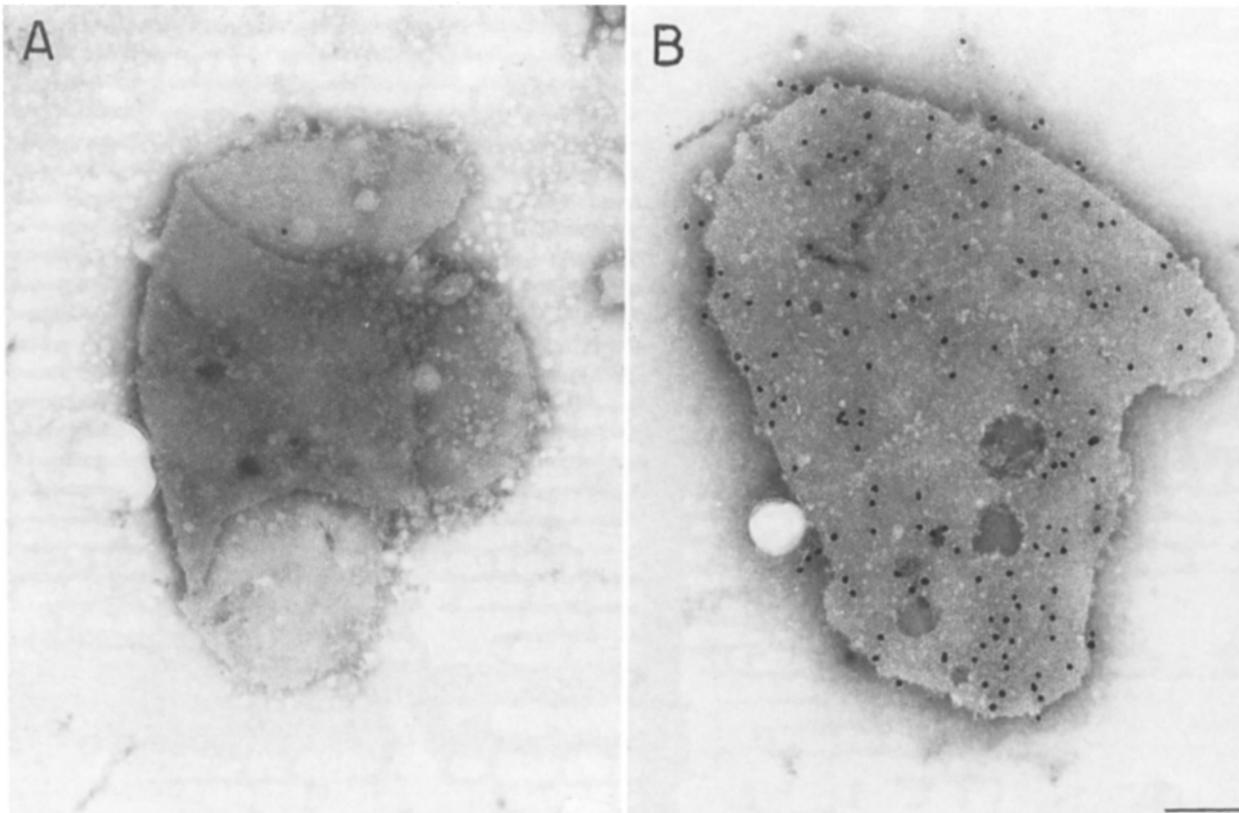
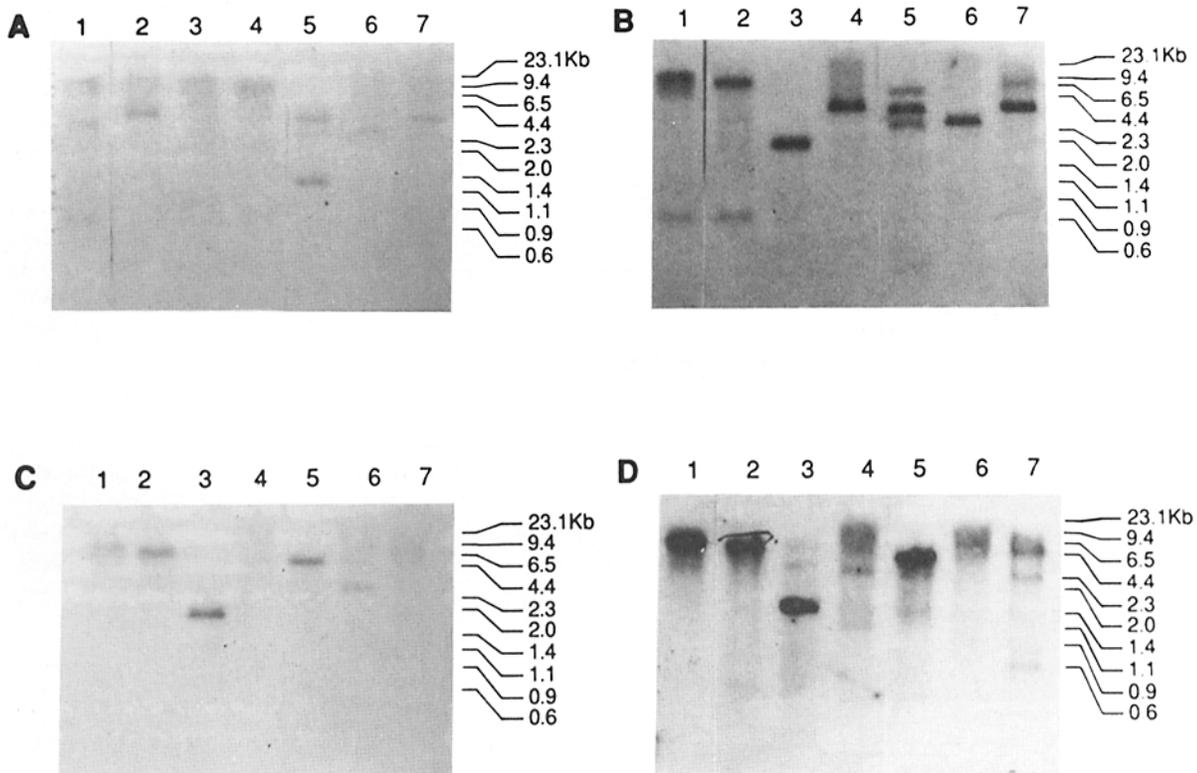


Figure 5. Electron microscopic immunolabeling of isolated gap junctions. Isolated mouse liver gap junctions were laid on a grid and incubated with affinity-purified preimmune (A) or immune (B) serum and then with anti-rabbit IgG conjugated to 10-nm gold particles. The primary antibody was raised against amino acids 101-119 of Cx26. All structures labeled by the immune serum were identifiable as gap junctions by their distinctive pattern of closely packed connexons. Nonjunctional membranes and cytoskeletal elements in crude junctional preparations were not labeled by this antibody under these conditions (data not shown). Bar, 0.1 μ m.



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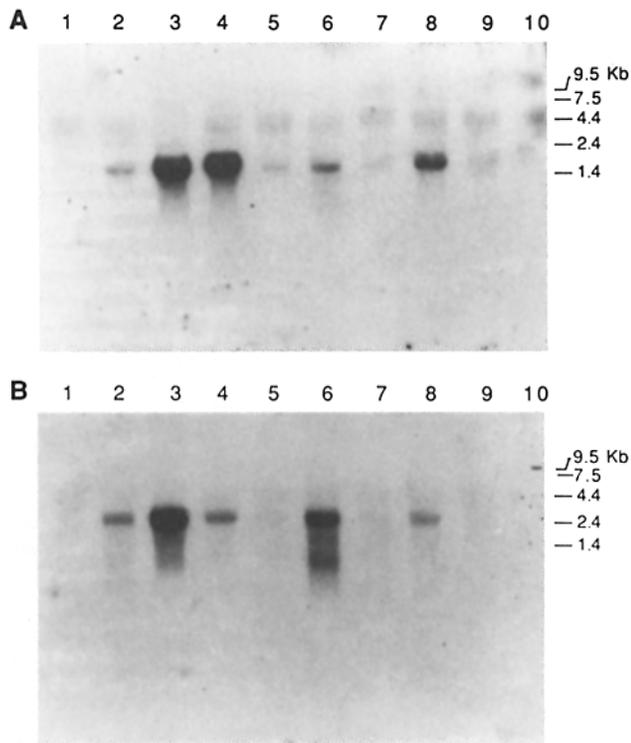


Figure 7. Hybridization of nick-translated Cx32 and Cx26 cDNA probes to total RNA purified from a number of different tissues. 20 μ g of total RNA purified from (lanes 1–10, respectively) rat heart, intestine, mouse liver, rat liver, lung, kidney, spleen, stomach, testis, and skeletal muscle by banding in CsCl (Chirgwin et al., 1979; Fryberg et al., 1980) were treated with glyoxal, separated on a 1% agarose gel, and transferred onto Gene Screen Plus membrane (DuPont Co.). The blots were preincubated and hybridized at high stringency (as described in Materials and Methods) with a nick-translated DNA probe from Cx32 (A) or Cx26 (B). Mobilities of RNA standards (Bethesda Research Laboratories) are shown on the right. All bands detected have characteristic mobility (1.6 kb for Cx32 and 2.5 kb for Cx26), except for some small components detected by Cx26 in kidney (lane 6). Some background from the 28S rRNA band is noticeable with Cx32.

and Fasman (1978) and Garnier et al. (1978). As in the other connexins studied to date, the third of these spans, when modeled as an α helix, displays a marked amphipathic character, with adjacent acidic and basic residues (Glu¹⁴⁷ and Arg¹⁴⁹) on the polar face. The two cysteine-rich domains (Fig. 8, B and D) demonstrated to be extracellular in Cx32 (Goodenough et al., 1989; Zimmer et al., 1987) and Cx43 (Yancey et al., 1989) are also conserved in Cx26 with an identical distribution of cysteine residues. By these criteria, in conjunction with the demonstrated ability of Cx32 (Young et al., 1987; Dahl et al., 1987) and Cx43 (Swenson et al., 1989) to form functional intercellular channels, it seems likely that Cx26 plays an integral role in forming aqueous pores be-

tween cells and is not merely an accessory protein of the gap junction. This conclusion is consistent with the x-ray analysis of isolated mouse liver gap junctions (Makowski et al., 1977) which shows no evidence of cytoplasmic or extracellular accessory material. Similar preparations have been demonstrated both biochemically (Nicholson et al., 1987) and immunologically (Traub et al., 1989; Fig. 5) to contain significant levels of Cx26 ($\geq 30\%$ of the total protein).

One question that remains at this point is whether Cx26, like Cx32 and Cx43, can form functional homopolymeric channels or whether it can only do so when oligomerized with other connexins, most notably Cx32. Anti-peptide antibodies specific to Cx26 uniformly label mouse gap junctional plaques (Fig. 5), producing a pattern of gold decoration indistinguishable from that produced by a Cx32-specific antibody (data not shown). Thus, we conclude, as have Traub et al. (1989), that no subdomains of either protein exist within a given gap junctional plaque. This coexistence of Cx32 and Cx26 also extends to the whole tissue level, where we have consistently observed coexpression of the messages for the two proteins to be the rule, albeit in varying ratios (Fig. 7). Subsequent analyses have revealed some cases where Cx26 is expressed with Cx43 rather than Cx32 (i.e., leptomeninges and pineal gland; our unpublished observations), but no reliable demonstration of Cx26 expression alone has yet been made. While the consistency of these results may be striking, a true assessment of their significance with respect to channel structure will require the analysis of each organ for connexin expression by cell type.

Along with the aforementioned similarities in both the structures and expression patterns of Cx32 and Cx26, Southern blot analyses (Fig. 6) indicate that the Cx26 gene has similar characteristics to that encoding Cx32 (Miller et al., 1988). Both are present as a single copy per haploid genome. The Cx26 gene also appears to lack introns of detectable size within the coding region, although one has been tentatively identified just upstream of the initiator codon (compare Cx32 gene; Miller et al., 1988). A second intron within the last 500 bases at the 3' end of the Cx26 message is also indicated by the genomic restriction digests (Fig. 6). Clearly, confirmation and specific localization of these elements must await isolation of a genomic clone.

In contrast to these general similarities, several differences between Cx32 and Cx26 are worthy of note, both at RNA and protein levels. Upstream of the consensus initiation site of the Cx26 message, a pair of inverted repeats (nucleotides –28 to –36 and –13 to –5) define a potential hairpin loop (calculated stability of –8.8 kcal/mol; Tinoco et al., 1975) that contains a sequence of nine nucleotides complementary to the 3' end of the 18S rRNA. Difficulties encountered sequencing this region using Klenow polymerase suggest that such secondary structure can readily form. Analogous structures have been found in other eukaryotic mRNAs (Hagenbuchle et al., 1978; Lomedico et al., 1979; Cooke et al., 1980), including MIP 26 (Gorin et al., 1984) and Cx43.

Figure 6. Southern blot analysis with Cx26. Each panel represents a Southern blot of restriction digests of rat liver genomic DNA electrophoresed on 1% agarose gels. Blots were hybridized to nick-translated fragments of Cx26 cDNA: (A) 5'-Hinc II fragment (probe I); (B) Hinc II-Bst XI fragment (probe II); (C) Bst XI-Hind III fragment (probe III); (D) Hind III-3' end (probe IV). Each lane has $\sim 10 \mu$ g of DNA digested with (lanes 1–7, respectively) Bam HI, Eco RI, Hinc II, Hind III, Pst I, Sma I, and Xba I. Size markers in kilobases shown on the right are Hind III fragments of lambda DNA and Hae III fragments of ϕ X 174 DNA (Bethesda Research Laboratories).

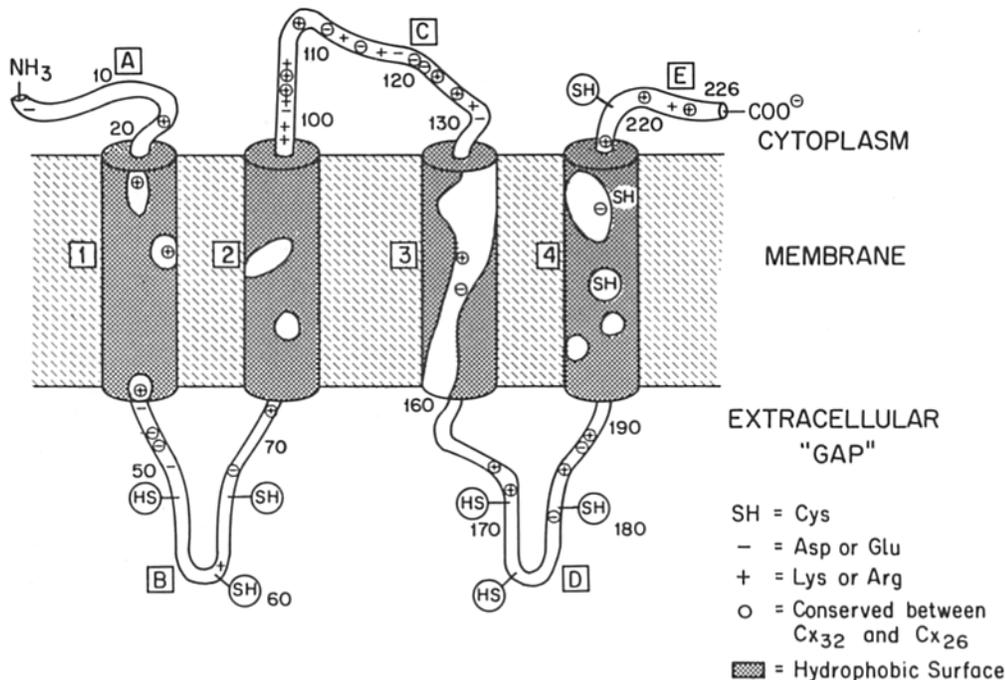


Figure 8. Topological model of the Cx26 gap junction protein. Transmembrane spans are modeled as α helices, with hydrophobic surfaces shaded and hydrophilic regions open. The distribution of basic (Lys and Arg [+]), acidic (Glu and Asp [-]) and Cys (SH) residues are specifically indicated. Circled symbols denote residues that are also found in Cx32. Hydrophilic and hydrophobic domains are labeled as A-E and 1-4, respectively, corresponding to the regions indicated in Fig. 3. (Reproduced from *Gap Junctions: Modern Cell Biology*, Vol. 7., 1988, 548 pp, by permission of Alan R. Liss, Inc., New York.)

Their similarity to the Shine-Delgarno sequences of prokaryotes has led to their implication in ribosomal binding of the message, thereby increasing translational efficiency. The absence of such a structure in the Cx32 message could explain why, in liver, the Cx26 protein is present in higher amounts with respect to Cx32 than would be predicted from a comparison of their message levels (Nicholson and Zhang, 1988).

At the protein level, the main divergence between Cx26 and other connexins occurs in domains A (amino terminus), C (central loop), and E (carboxy terminus), as indicated in Figs. 3 and 8. These domains, demonstrated to be cytoplasmic in the cases of Cx32 and Cx43, display 56, 32, and 21% amino acid identity between Cx26 and Cx32, compared with $\sim 75\%$ over the rest of the molecule (Fig. 3). The most notable difference is the truncation of the carboxy terminus of Cx26 to a length of only 11 residues (Figs. 3 and 8). This results in a paucity of potential regulatory elements compared with Cx32. An example of this is the lack, in Cx26, of consensus phosphorylation sites for cAMP-dependent protein kinase comparable with those found in Cx32 (i.e., serines 234, 241, and 280). This is consistent with results from primary hepatocyte cultures (Traub et al., 1989), as well as isolated junctions (Saez et al., 1986), in which Cx32 could be covalently tagged with ^{32}P , while Cx26 failed to take up label under the same conditions. To date, it remains undetermined as to whether potential targets for other kinases that are found on Cx26 (i.e., tyrosine kinase at Tyr⁹⁷ and Tyr²¹⁷ or Ca⁺⁺-dependent kinase at Ser²¹⁹) are used or if these agents serve to modulate channel function in the liver.

A clearer view of the molecular details of gap junction structure and the variants that occur is now beginning to emerge, but much is left to be done to refine our current crude and, of necessity, speculative models of junction structure. Specific sites controlling channel gating, interconnexon interaction, and channel structure all need to be identified in each gap junction subtype. The major question of whether

Cx32 and Cx26 form separate or heteropolymeric channels also remains unresolved. However, the isolation of these and other cDNA clones and their expression and reconstitution in different systems should soon help clarify many of these issues and should ultimately lead us to an understanding of the functional significance for the organism of such a diverse array of different intercellular channels.

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