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原　　著

Molecular Cloning of Two Guinea Pig Liver Gap Junction Proteins, Connexin32 and Connexin26

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Abstract Intercellular coupling of hepatocytes through gap junctions facilitates exchange of small metabolites or ions, and contributes to maintenance of tissue homeostasis. As protein constituents of the liver gap junction channels, connexin32 (Cx32) and Cx26 have been identified. By use of rat cDNA probes, we cloned cDNAs for guinea pig homologs of Cx32 and Cx26, and compared their amino acid sequences with those of other species. The deduced primary structure of guinea pig Cx32 was 283 amino acids long and contained 98% identical amino acids to the rat and human Cx32. Only six amino acid exchanges were detected between the guinea pig and rat Cx32. On the contrary, the deduced amino acid sequence of guinea pig Cx26 (226 amino acids long) was 91 and 89% identical to the rat and human Cx26, respectively. Twenty-one amino acid exchanges were found between the guinea pig and rat, and the divergence was mostly located in cytoplasmic domains of Cx26. These results suggest that Cx26 shows structural diversity between species, while Cx32 is highly conserved.

Key words: gap junction, connexin32, connexin26, intercellular communication, cDNA cloning, guinea pig, liver

Introduction

Gap junctions form clusters of transmembrane channels that mediate intercellular communication including electrical coupling and metabolic cooperation[1]. Each unit channel is a doughnut-shaped particle called a connexon, which is composed of a hexamer of connexin (Cx) proteins encircling a hydrophilic pore channel[14)25)].

To date, more than ten Cxs have been cloned in rodents[3)5)7)]. A variety of studies indicate that the Cxs belong to a multigene family of highly related proteins. In addition to a high level of primary sequence similarity, the Cx transmembrane topology is highly conserved. Hydropathy analysis predicts four transmembrane, two extracellular and three cytoplasmic domains, with N- and C-termini located in the cytoplasm[2)28)29)]. The extracellular, transmembrane and N-terminal cytoplasmic domains are well conserved among the family members, while the central and C- terminal cytoplasmic domains are highly variable in both sequence and size[8)]. The expression pat-

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terns of Cxs are characteristically specific for tissue and cell types[10)13)16)21)27)].

In the mammalian liver gap junctions, two Cx molecules, Cx32 and Cx26 have been identified[11)22)28)]. The relative content of these proteins varies among species. In SDS-PAGE analysis of isolated liver gap junctions, relative protein ratios of Cx32 to Cx26 are 2 : 1 in mouse, 10 : 1 in rat, and 2.3 : 1 in rabbit[18)23)]. In contrast, the major component in the guinea pig liver gap junctions is Cx26, with about a threefold higher content than Cx32[23)]. Double-labeling immunofluorescence microscopy has revealed that Cx32 and Cx26 were localized together in the same gap junction plaques in the guinea pig liver[12)] as well as in mouse[18)] and rat[24)] livers. At the level of electron microscopic resolution, Cx32 and Cx26 were randomly intermixed within a single gap junction plaque of the guinea pig and rat livers[12)].

Amino acid sequences of Cx32 and Cx26 have been deduced from the cDNA of the rat[11)22)28)], human[11)15)] and from the genomic DNA of the mouse[9)]. However, no sequence data of the guinea pig Cxs is available until now. In this study, by cDNA cloning, we intended to characterize the guinea pig homologs of Cx32 and Cx26 at their amino acid sequence levels. By sequence comparison of Cx26 between species, the results suggested some structural diversity of cytoplasmic domains of Cx26.

Materials and Methods

Isolation of cDNA clones

A guinea pig liver cDNA library constructed in the λgt10 vector (Clontech) was screened by using either rat Cx32 cDNA[22)] or Cx26 cDNA[28)] as described elsewhere[17)20)].

DNA sequencing

DNA fragments were subcloned into multiple cloning sites of the pUC119 vector and sequenced by the dideoxy-chain termination method in both directions. The nucleotide sequences determined were compiled and analyzed with the DNASIS programs (Hitachi Software Engineering Co. Ltd.).

Total cellular RNA preparation

Two adult female Hartley strain guinea pigs weighing 350-380 g were permitted to feed and drink ad *libitum*. Animals were deeply anesthetized with ether, and the livers were quickly excised and frozen in the liquid nitrogen. All experimental procedures using animals followed the Guidelines for animal experiments of the Faculty of Medicine, Kyushu University.

Total cellular RNA was prepared as described previously[26)] with some modifications. Frozen livers were pulverized and homogenized with 10 volumes of homogenization buffer using a Polytron homogenizer for 90 sec at room temperature. The homogenization buffer consisted of RNA-extraction buffer (0.1 M Tris-HCl, pH 8.0, 0.1 M NaCl, 5 mM EDTA, 2 % SDS) and an equal volume of a mixture of organic solvents (phenol : isoamyl alcohol ; 100 : 1 in volume). After centrifugation at 1680g for 20 min at room temperature, the aqueous phase was separated and further homogenized with an equal volume of phenol. The homogenization and separation step was repeated four times. Finally, the aqueous phase was homogenized with the organic solvent mixture supplemented with chloroform (phenol : chloroform : isoamyl alcohol ; 100 : 100 : 1 in volume) and separated as above. RNA was precipitated by adding ethanol. The pellet was dissolved in 20 mM EDTA and precipitated again with 3 M

Na acetate, pH 5.2. The RNA precipitate was rinsed with cold 85% ethanol and dried under vacuum. RNA was finally dissolved in distilled water, quantified by absorbance at 260 nm, and stored at −80°C until use.

Northern blot analysis

Total cellular RNA (20 μg/lane) was electrophoresed in 1.5% agarose/formaldehyde gels containing 0.5 μg/ml ethidium bromide, and transferred overnight onto nylon membranes (Hybond N ; Amersham). After baking under vacuum for 2 hrs at 80°C, the filters were prehybridized in 5 × standard saline citrate (SSC), 5×Denhardt's solution, 0.5% SDS, 100 μg/ml fish sperm DNA, 50% formamide for 2 hrs at 42°C, then hybridized with ^{32}P-labeled cDNA probe in the same buffer overnight at 42°C. For hybridization, a *Pst*I-*Eco*RI fragment of guinea pig Cx32 cDNA (representing the full coding sequence) and a *Eco*RI fragment of guinea pig Cx26 cDNA (representing 50% of the coding region) were used. After hybridization, the filters were washed twice in 2× SSC, 0.1% SDS for 10 min each, once at room temperature and once at 42°C, and washed twice in 1 × SSC, 0.1 % SDS for 10 min at 65°C. Autoradiography was carried out with imaging plates and analyzed with a BAS 2000 Bio Image Analyzer (Fuji).

Nucleotide and amino acid sequence accession numbers

The DDBJ-GenBank-EMBL accession numbers for the guinea pig Cx32 and Cx26 nucleotide and amino acid sequences are AB089439 and AB089440, respectively.

Results

Cloning and sequence analysis of the guinea pig Cx32

A single positive signal on duplicate filters was obtained by screening 400,000 plaques of a guinea pig cDNA library at high stringency condition with the ^{32}P-labeled rat Cx32 cDNA. This plaque was purified to homogeneity and a resulting DNA fragment was subcloned for sequence analysis. The nucleotide sequence, together with the deduced amino acid sequence of the coding region is shown in Fig. 1. The cDNA clone was 1,357 bases long and had a single long open reading frame which encodes a putative protein of 283 amino acid residues with a predicted molecular mass of 32,010 dalton. The overall nucleotide homology between guinea pig and rat Cx32 was 90.6% within the open reading frame. The amino acid sequence deduced from the guinea pig Cx32 cDNA contained 97.9% identical amino acids to the rat or mouse Cx32[9)22)] and 97.5% identical amino acids to the human Cx32[11)]. Of the 283 amino acids, there were six amino acid differences between guinea pig and rat. These substitutions are indicated in Fig. 1. Three of the six amino acid exchanges were found in the putative transmembrane and extracellular domains at positions 67, 171 and 189. The other three exchanges were detected in the C-terminal cytoplasmic domain (positions 223, 234 and 273), while no amino acid exchange was found in the N-terminal or central cytoplasmic domains.

Cloning and sequence analysis of the guinea pig Cx26

The ^{32}P-labeled rat Cx26 cDNA fragment were used to screen 300,000 bacteriophage plaques from the guinea pig cDNA library under high stringency condition. Three consistently positive clones were isolated. The nucleotide sequences of all the clones were determined on both strands; they overlapped with no discrepancies. The

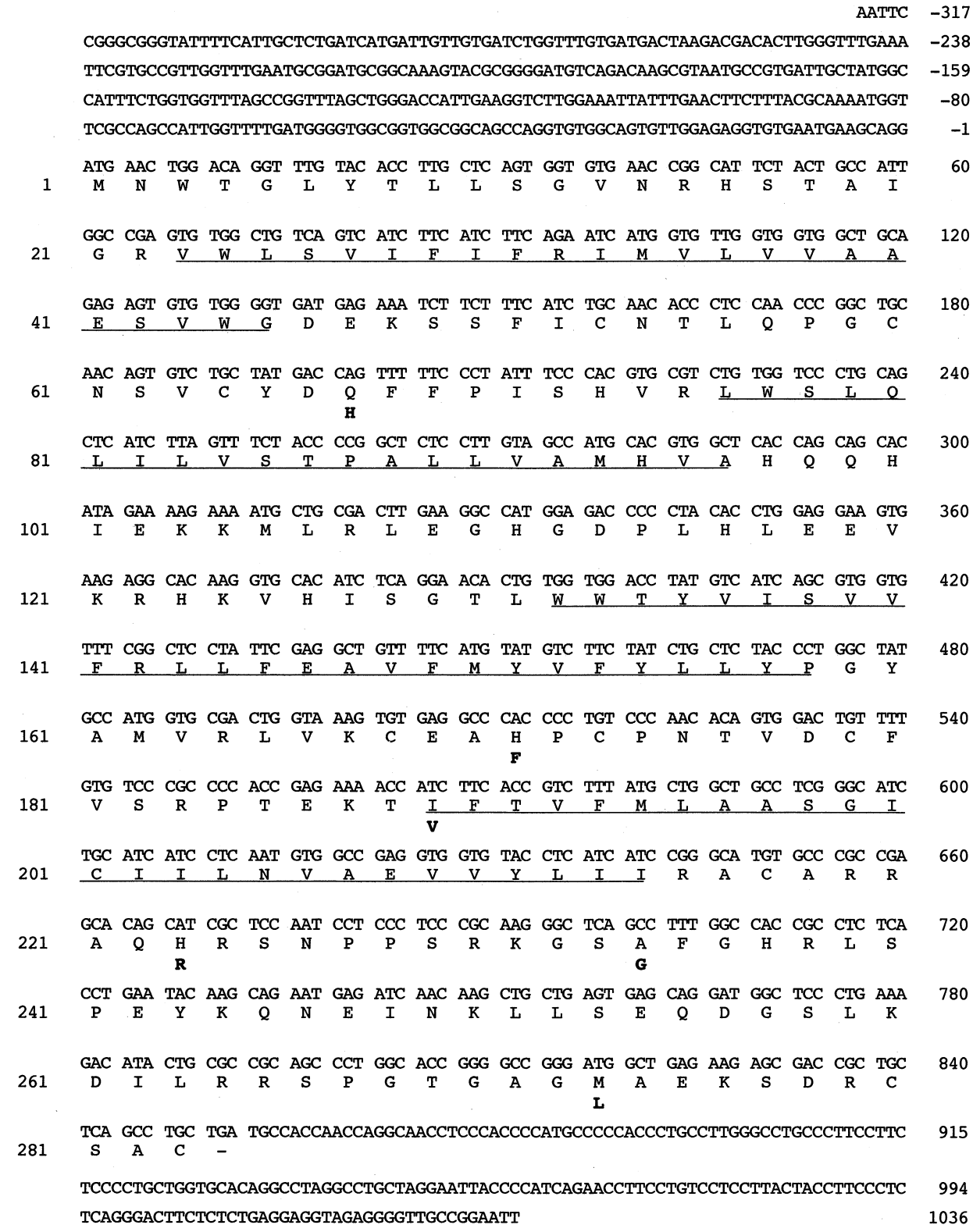

Fig. 1 Nucleotide and deduced amino acid sequence for the guinea pig Cx32 cDNA. Numbers to the left and right of the sequences show the positions of amino acids and nucleotides, respectively. Four hydrophobic, putative transmembrane domains are underlined. The amino acid differences in the rat sequence are shown below the guinea pig sequence in boldface letters.

composite cDNA sequence was 926 bases long and covers the entire Cx26 coding region which is contained within a single open reading frame of 678 nucleotides (Fig. 2). The overall nucleotide homology between guinea pig and rat Cx26 was 85.9% within the open reading frame. The derived amino acid sequence was 226 amino acids long and coded for a protein with a calculated molecular mass of 26,487 dalton. The amino acid sequence was 90.7, 89.8, and 89.4% identical to the derived amino acid sequences from rat[28], mouse[9], and human[15] Cx26 cDNAs, respectively. Of the 226 amino acid residues, there were 21 amino acid differences between guinea pig and rat. Within the amino acid sequence of guinea pig Cx26, homology with those of other species was not uniform. Fig. 3 shows an alignment of the Cx26 amino acid sequences of the four species as mentioned above. In the three putative cytoplasmic domains (designated as domains a [amino acids 1-22], c [amino acids 97-132], and e [amino acids 216-226] in Fig. 3, also see Fig. 5), the rat Cx26 shows 97-100% amino acid identity to the mouse Cx26 and 91-95% identity to the human Cx26. In these domains, however, the guinea pig Cx26 shared amino acid homologies of 78-82% with the rat and mouse Cx26, and 82-91% with the human Cx26. Intervening sequences

```
                                                                CAGTACCAACCTCTCAGTGGACAGG    -1
    ATG GAT TGG GGT GCG CTC CAT GCG ATC CTG GGA GGT GTC AAC AAA TAC TCC ACC AGT ATC    60
1    M   D   W   G   A   L   H   A   I   L   G   G   V   N   K   Y   S   T   S   I

    GGG AAG ATC TGG CTC ACG GTC CTT TTC ATT TTT CGA GTC ATG ATC CTC GTG GTG GCC ACT   120
21   G   K   I   W   L   T   V   L   F   I   F   R   V   M   I   L   V   V   A   T

    AAG GAG GTG TGG GGA GAT GAG CAG GCT GAT TTT ACC TGC AAC ACC CTC CAG CCT GGC TGC   180
41   K   E   V   W   G   D   E   Q   A   D   F   T   C   N   T   L   Q   P   G   C

    AAA AAT GTG TGC TAT GAC CAC TAC TTC CCC ATC TCG CAC ATC CGG CTG TGG GCC CTG CAG   240
61   K   N   V   C   Y   D   H   Y   F   P   I   S   H   I   R   L   W   A   L   Q

    CTG ATC TTT GTG TCC ACG CCG GCA CTC CTG GTG GCC ATG CAC GTG GCC TAC CGG AAG CAT   300
81   L   I   F   V   S   T   P   A   L   L   V   A   M   H   V   A   Y   R   K   H

    GAG AAG AAA AGG AGG TTC ATT AAA GGA GAG ATG AAG AGC GAA TTC AAG GAC ATC GAG GAG   360
101  E   K   K   R   R   F   I   K   G   E   M   K   S   E   F   K   D   I   E   E

    ATC AAA AAC GAG AAG ATC CGC ATA GAA GGG TCC CTG TGG TGG ACC TAC ACC ACC AGT ATC   420
121  I   K   N   E   K   I   R   I   E   G   S   L   W   W   T   Y   T   T   S   I

    TTC TTC CGG GTC ATC TTC GAA GGT GCC TTC ATG TAT GTG TTT TAC ATC ATG TAC AAC GGC   480
141  F   F   R   V   I   F   E   G   A   F   M   Y   V   F   Y   I   M   Y   N   G

    TTC TTC ATG CAG CGC CTG GTG AAA TGC AAT GCT TGG CCT TGC CCC AAC ACG GTG GAC TGC   540
161  F   F   M   Q   R   L   V   K   C   N   A   W   P   C   P   N   T   V   D   C

    TTT ATC TCC AGG CCC ACA GAG AAG ACT GTC TTT ACT GTG TTC ATG ATT GTG GTG TCA GGC   600
181  F   I   S   R   P   T   E   K   T   V   F   T   V   F   M   I   V   V   S   G

    ATT TGC ATC TTG CTA AAC ATC ACA GAA TTG TGT TAT TTG TTC ATT AGA TAT TGT TCC AAA   660
201  I   C   I   L   L   N   I   T   E   L   C   Y   L   F   I   R   Y   C   S   K

    AAG TCC AAA AAA CCT GTT TAG CATTGCTCAGCTATAGATAAAAGATGAAGTGACCTGTGTTTAGCTGTCAAG   732
221  K   S   K   K   P   V   -

GCTCAGTCACCAGCATTTGGCAAGACAAAGATTCTCATCTTAATTTAACCATAGAACCCCTAGGCTCCAGGAAACCAGG   811

TGCTGTATGGGGGCAAGTCCCAGAGCCCAAACAAGTCAATGTGTAATGTTAATAAATAGTCCAATGAGACCGCCTGCTG   890

GTATTGCGTAA                                                                        901
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Fig. 2 Nucleotide and deduced amino acid sequence for the guinea pig Cx26 cDNA. Numbers to the left and right of the sequences show the positions of amino acids and nucleotides, respectively.

which contain both extracellular and transmembrane domains (coding amino acids 23-96 and 133-215) of the guinea pig Cx26 showed 93-96% amino acid homologies to the rat and mouse Cx26. The potential consensus sites for kinases were conserved in guinea pig Cx26 protein (Tyr^{97} and Tyr^{217} for tyrosine kinase, Ser^{219} for Ca^{2+}-dependent kinase)[28].

Northern blot analysis

To confirm the expression of identified sequences, we performed Northern blot analysis of total cellular RNA prepared from the adult guinea pig liver (Fig. 4). Guinea pig Cx32 and Cx26 cDNAs strongly hybridized to bands at the positions corresponding to 1.6 kb and 2.4 kb, respectively.

Discussion

In this study, we have cloned and sequenced the guinea pig homologs of Cx32 and Cx26 which are identified previously in liver gap junctions. Northern blot analysis has comfirmed their strong mRNA expression in the guinea pig liver.

The amino acid sequence of the guinea pig Cx32 is highly homologous to those of mouse, rat, and human. In the Cx32 protein, C-terminal cytoplasmic domain is thought to be potential regulatory elements. Though three amino acid exchanges are found in the domain of the guinea pig Cx32, two potential cAMP dependent phosphorylation sites (Ser^{233} and Ser^{240})[11] are conserved in all the species examined. Interest-

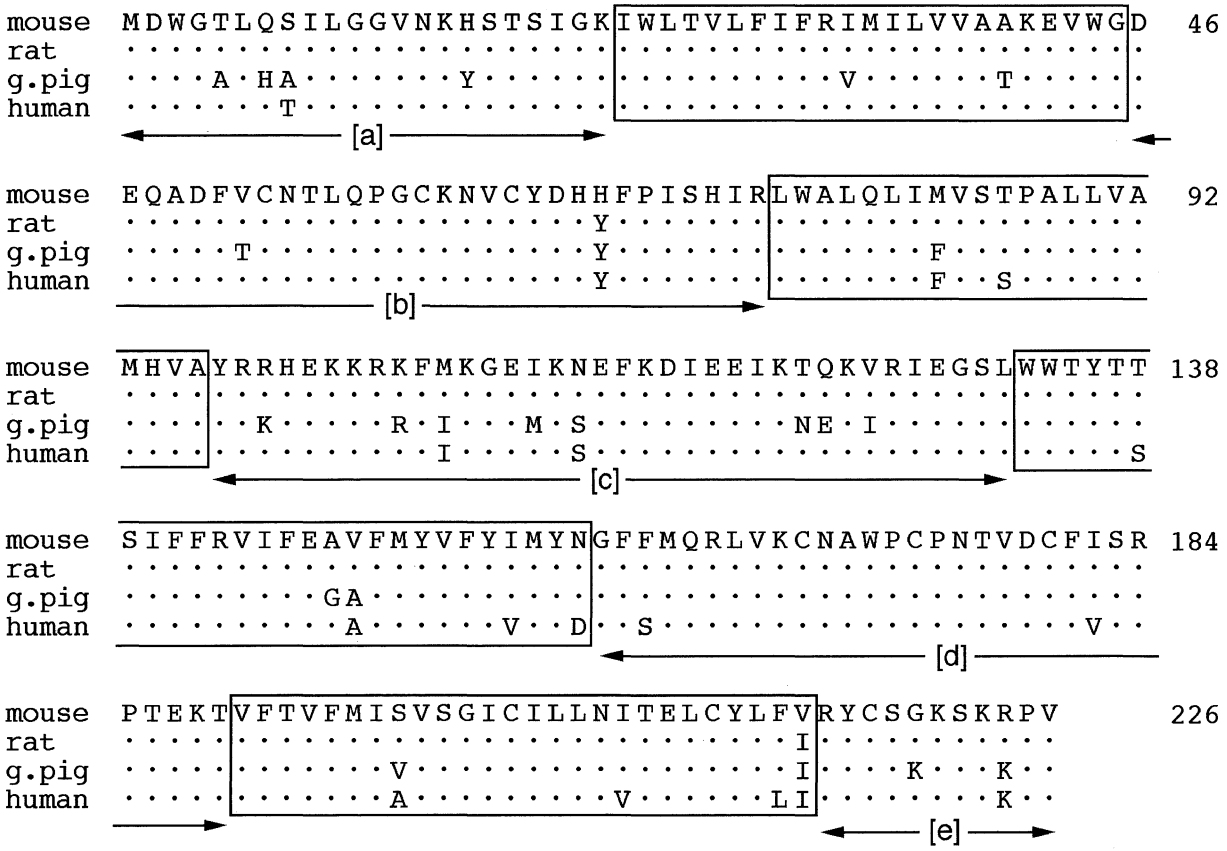

Fig. 3 Comparison of amino acid sequences of the mouse, rat, guinea pig (g.pig), and human Cx26 proteins. Numbers to the right of the sequences show the positions of amino acids. Dots indicate identical amino acids to those in the mouse sequence. [a], [c], and [e] are proposed to be cytoplasmic domains. [b] and [d] are proposed to be extracellular domains. Four hydrophobic, putative transmembrane domains are shown in boxes.

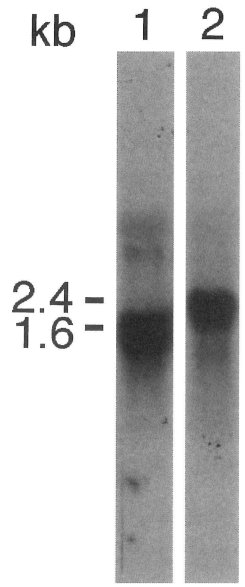

Fig. 4 Northern blot analysis of Cx32 and Cx26 mRNA transcripts in the guinea pig liver. Total cellular RNAs (20 μg/lane) are electrophoresed on a 1.5% formaldehyde-agarose gel, transferred to a nylon membrane, and probed at high stringency with the guinea pig Cx32 cDNA (Lane 1) and Cx26 cDNA (Lane 2). Note that the prominent bands are with characteristic mobility (1.6 kb for Cx32 and 2.4 kb for Cx26). Some background from the 28S rRNA band is noticeable with the Cx32 cDNA probe (Lane 1).

ingly, comparison of amino acid sequences between species including human, in the N-terminal and central cytoplasmic domains as well as in the four transmembrane domains, has revealed that the sequences matched completely except for only one amino acid substitution in the guinea pig sequence at position 189 : Ile (guinea pig) / Val (mouse, rat and human). Thus, the primary structure of Cx32 seems to be highly conserved between species. This fact may suggest the functional importance of Cx32 in the role of intercellular communication.

On the contrary, Cx26 shows much more sequence divergence between species than Cx32, though its basic structural characteristics are conserved. Overall sequence homology between the guinea pig and rat Cx26 protein is 90.7%. This value is lower than the homology between the rat and human Cx26 (93.4%). Furthermore, sequence comparison has revealed that the divergence between guinea pig and other species is mostly present in the cytoplasmic domains (Figs. 3 and 5). When compared to the amino acid sequence of rat Cx26, 67% (14/21) of the substituted amino acids are located in the cytoplasmic domains of guinea pig Cx26. In the case of human Cx26, the corresponding is only 27% (4/15). Among the Cx family, the most notable structural feature of Cx26 is truncation of the C-terminal cytoplasmic domain to a length of only 11 amino acid residues. This seems to result in a lack of potential regulatory elements[28]. However, recent reports have suggested that each Cx subtype forms channels which are characterized by different selective permeability[4)6)19]. Since cytoplasmic domains of Cx are located near the entrance or exit of gap junction channels, there is still a possibility that the N-terminal or central cytoplasmic domains of Cx26 work as a manager of passing molecules. The structural divergence of Cx26 protein may serve as a key-point to solve the question.

By analysis of the promotor regions of mouse Cx32 and Cx26 genes, Hennemann et al. have suggested different regulatory mechanisms for transcription of each Cx gene[9]. Abundant expression of Cx26 protein in the guinea pig liver may be attribut-

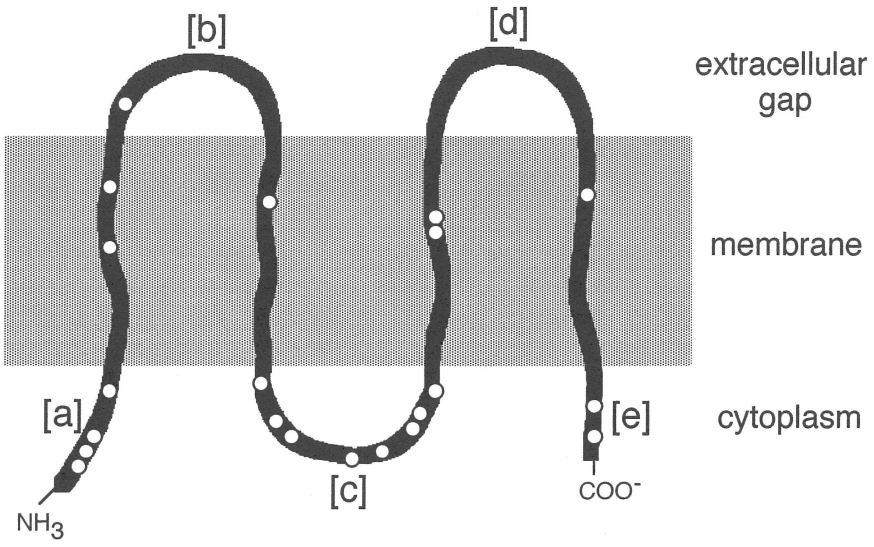

Fig. 5 Topological model of the guinea pig Cx26 gap junction protein based on the hydropathy plots by Zhang and Nicholson (1989) and orientation presented by Zimmer et al. (1987). The distribution of twenty-one amino acid exchanges between guinea pig and rat are indicated by white dots. Hydrophilic domains are labeled as [a]-[e], corresponding to the regions indicated in Fig. 3. Note that the changed amino acids are mainly found in the cytoplasmic domains ([a], [c], and [e]).

ed to this point. Further studies are being performed to identify the promotor sequences of guinea pig Cx32 and Cx26 genes.

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(和文抄録)

モルモット肝ギャップ結合構成蛋白・コネキシン32およびコネキシン26の遺伝子クローニング

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九州大学大学院医学研究院耳鼻咽喉科学[2)]
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倉岡晃夫[1)]・中島寅彦[2)]
川渕優[1)]・柴田洋三郎[3)]

肝ギャップ結合を介する細胞間コミュニケーションは，代謝産物やイオンの交流を通し，組織内ホメオスタシスの維持に寄与する．肝細胞間ギャップ結合の構成蛋白としてコネキシン32（Cx 32）とコネキシン26（Cx 26）が同定されているが，今回我々はラットcDNAプローブを用い，モルモット由来のCx 32およびCx 26の遺伝子クローニングを行い，推定されるアミノ酸配列の比較検討を行った．モルモットCx 32は283個のアミノ酸から構成され，ラットおよびヒトCx 32とのアミノ酸配列の相同性は98%に達した．モルモットとラット間では6個のアミノ酸置換が認められるのみであった．一方，モルモットCx 26は226個のアミノ酸から構成され，ラットおよびヒトCx 26とのアミノ酸配列の相同性はそれぞれ91%，89%であった．モルモットとラット間でのアミノ酸の置換数は21個であり，多くの置換はCx 26の細胞質側領域に集中していた．以上の結果より，動物種間におけるCx 32アミノ酸配列の高度な保存性，ならびにCx 26の構造的多様性が示唆された．