Differences in expression patterns between mouse connexin-30.2 (Cx30.2) and its putative human orthologue, connexin-31.9[1]

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Abstract **A novel gap junction forming protein, mouse connexin-30.2 (Cx30.2) contains 278 amino acid residues, and is 79% identical to human Cx31.9 (GJA11). Northern analysis showed that *Cx30.2* is ubiquitously expressed, most prominently in testis. Polyclonal antibodies against Cx30.2 detected a 30 kDa protein in cells overexpressing *Cx30.2*, and in mouse testis. Immunofluorescence showed that Cx30.2 was expressed in vascular smooth muscle, but also in cell types where Cx31.9 was not detected. These data demonstrate that Cx30.2 is a bona fide gene, and suggest that it is the orthologue of Cx31.9, but that it may have additional roles compared with Cx31.9.**

Key words: Connexin; Gap junction; Connexin-30.2; Connexin-31.9; Smooth muscle; Mouse

1. Introduction

Gap junctions are channels linking the cytoplasm of neighboring cells, which enable the diffusion of small molecular weight substances that are important for many cellular activities, including coordination of cellular behavior [1]. A gap junction channel is formed by the docking of two hemichannels (connexons) from opposing cells. Each connexon consists of six connexin (Cx) subunits, which are the sole proteins required to form functional gap junctions. A connexin spans the plasma membrane four times, and contain two extracellular loops, a cytoplasmic N-terminal region, a cytoplasmic loop, and a cytoplasmic C-terminal region [2]. The most significant differences between connexin isoforms are the lengths and sequences of the two latter regions. Different connexin isoforms have different expression patterns and channel characteristics, which is thought to be important to regulate the diffusion of specific small molecules [1]. The connexin gene family has been reported to contain at least 20 members in human and 19 in mouse [3,4]. The proteins encoded by many of these genes have been characterized functionally, and their expression patterns have been analyzed, but a number of novel genes have only recently been identified by sequence similarity, and it remains to be determined if these are expressed in vivo, and if they encode functional connexins. We, and others, have recently cloned and characterized one of these novel connexin genes, human *Cx31.9*, and shown that it encodes a functional gap junction protein with unique channel properties [5–7]. Cx31.9 clustered together with the α-group of connexins by phylogenetic analysis, and was also designated α11 connexin (GJA11).

Antibodies raised against Cx31.9 showed that this connexin is mainly expressed in vascular smooth muscle cells [5]. Normal vascular function is dependent on coordination of vascular responses, which is thought to involve cell to cell coupling via gap junctions [8]. In vascular cells, gap junctions are found between endothelial cells, smooth muscle cells, and between the two cell types. Besides Cx31.9, at least four other connexin isotypes have been previously reported in vascular tissues [8]. The diversity of connexins in vascular tissue is thought to be important for the regulation of vessel relaxation and contraction. This was illustrated by the perturbation of one of these connexins in the vascular system (Cx40), which was shown to result in altered blood pressure in mice [9].

We here report the cloning and expression pattern of a putative mouse orthologue of Cx31.9, designated Cx30.2. Similar to Cx31.9, Cx30.2 is expressed in vascular smooth muscle cells, but was also detected in cell types not expressing Cx31.9.

2. Materials and methods

2.1. Genomic cloning and RACE

A 129/Sv mouse λ FIX® genomic library (Stratagene) was screened with a probe encompassing the *Cx31.9* open reading frame at low stringency. Several hybridization positive clones were identified, λ DNA purified, restriction digests were screened by Southern blotting using the Cx31.9 probe, and hybridization positive fragments sequenced. From a single genomic clone, a 4981 bp *Eco*RV fragment was subcloned to pGEM, and sequenced at the ends and with gene specific primers and primer walking. This fragment contained an open reading frame encoding Cx30.2, and 27 bp downstream. To obtain further downstream sequence, rapid amplification of 3′ ends (3′RACE) was performed using a commercial kit containing cDNAs from mouse brain, with anchor oligonucleotides ligated to the 3′ and 5′ ends (Marathon-Ready™ cDNA, Clontech). A primer from the

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[1] The nucleotide sequence of the mouse connexin-30.2 coding region, and surrounding genomic sequence, has been deposited in the GenBank/EMBL databases under GenBank accession number AY166870.

Abbreviations: Cx, connexin; eGFP, enhanced green fluorescent protein; UTR, untranslated region; RACE, rapid amplification of 3′ or 5′ ends; wt, wild type; pAb, polyclonal antibody; IF, immunofluorescence; mAb, monoclonal antibody

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3′ end of the Cx30.2 open reading frame, pn115 (5′-GTCGCCCTC-TTTGCCATCGCAG-3′), and Clontech primer AP1 were used for first round amplification, while nested primers pn116 (5′-CCCTTTC-CCGCCAGCCTACG-3′) and AP2 were used for the subsequent amplification. The resultant polymerase chain reaction (PCR) products were cloned into pGEM T/A cloning vector (Invitrogen) and sequenced. A 550 bp product was excised from the vector and used to rescreen restriction fragments of the genomic λ clone, and an approximately 8 kb *Eco*RV fragment was identified, subcloned to pGEM7, and sequenced. From this clone, 5930 bp contained mouse genomic DNA, while the remaining downstream sequence contained λ DNA. A *Sal*I restriction site indicated the border between the two segments. When compared with the 3′RACE products, this clone was found to contain the downstream sequence of the Cx30.2 gene. To obtain further genomic sequence upstream of the 4981 bp *Eco*RV fragment, a 4320 bp *Bgl*II fragment from the λ clone was identified, and found to contain 1467 bp further upstream of the *Eco*RV site. This fragment was cloned to pGEM7 and sequenced.

2.2. Northern blot analyses

The 550 bp 3′RACE fragment, from the 3′ Cx30.2 coding sequence and 3′ untranslated region (UTR), was random prime labeled with ^{32}P, purified, and hybridized to a Clontech mouse MTNI blot at 68°C, followed by washing to a stringency of 0.1×SSC at 60°C.

2.3. Generation of polyclonal antibodies (pAbs)

Regions of the Cx30.2 gene encoding parts of the putative cytoplasmic loop (amino acid residues 95–127), and the C-terminus (amino acid residues 229–278) were amplified by PCR using the following primers: Loop: pn147 (5′-ATGGGATCCTACTCCATGCACCAG-GCCAGC-3′), pn148 (5′-ATGGAATTCCTACAGGGCGCACGG-GGAGCACGG-3′). C-terminus: pn149 (5′-ATGGGATCCTCGCC-CTCTTTGCCATCGCAG-3′), pn150 (5′-ATGGAATTCCTAGAT-GGCCAGGTCCTGGCG-3′). The amplicons were digested with *Bam*HI and *Eco*RI, subcloned into pGEX2TK (Amersham Pharmacia), and sequenced. Glutathione *S*-transferase (GST) fusion proteins containing the connexin domains were induced using standard procedures, and purified using glutathione-agarose (Sigma). Eluted fusion proteins were dialyzed against distilled water, lyophilized, and used in 500 μg aliquots to immunize rabbits. Rabbit sera were affinity purified against the respective fusion proteins cross linked to glutathione-agarose columns, according to the instructions from the manufacturer (GST Orientation kit, Pierce). Antibodies eluted from the affinity columns were concentrated to 1–10 mg/ml.

2.4. Expression constructs

Cx30.2 GFP construct: primers pn145 (5′-ATGGAATTCAGCGT-CATGGGGGAGTGGG-3′) and pn146 (5′-ATGGGATCCCGGA-TGGCCAGGTCCTGGCGC-3′) were used to amplify the Cx30.2 open reading frame by PCR using the Expand Hi Fidelity PCR system (Roche Molecular Biochemicals). The PCR product was digested with *Eco*RI and *Bam*HI, and cloned into expression vector eGFPN-1 (Clontech), to generate an open reading frame encoding mCx30.2 with a COOH-terminal enhanced green fluorescent protein (eGFP) tag. The vector, eGFP-N1, contains a CMV promoter and a SV40 polyadenylation signal.

Untagged Cx30.2 construct: an *Eco*RI-*Eco*RV 1225 bp fragment from the 4981 bp λ fragment (containing 369 bp upstream sequence, and the full Cx30.2 open reading frame) was subcloned into pBluescript II, and then into *Eco*RI-*Xho*I linearized expression vector pCDNA3 (Invitrogen).

2.5. Protein extraction and immunoblot analyses

Tissues and cells were homogenized in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer, and used in immunoblot analysis as previously described [4].

2.6. Cell culture and confocal laser scanning immunofluorescence

HEK-293 (human embryonic kidney) cells were transfected with Cx30.2 constructs, and processed for immunofluorescence (IF) as previously reported [5]. Mouse tissues were cut from frozen blocks, fixed in 4% paraformaldehyde at room temperature for 20 min, and processed as previously reported. Smooth muscle cells were detected using monoclonal antibody (mAb) 1A4 (anti-α-smooth muscle actin, Dako-Cytomation). Rabbit pAbs were detected with fluorescein isothiocyanate-conjugated goat anti-rabbit (Southern Biotechnology Associates), and 1A4 with Texas red goat anti-mouse. This secondary antibody gave high background on mouse tissues, but it was possible to distinguish the smooth muscle cells. Controls included stainings with each primary and each secondary antibodies separately, and using irrelevant primary pAbs and mAbs.

3. Results and discussion

3.1. Molecular cloning of mouse Cx30.2 and analysis of the protein sequence

In search for a mouse orthologue of human Cx31.9 [5], a mouse 129/Sv genomic library was screened with a probe encompassing the coding region of Cx31.9 at low stringency. Several hybridization positive clones were identified, and restriction fragments sequenced. From one genomic clone, a 4981 bp *Eco*RV fragment was found to contain an open reading frame encoding a 278 amino acid protein with a predicted molecular mass of 30 262 (Fig. 1). Hydropathy analysis of this putative protein revealed the presence of four potential transmembrane regions, similar to other connexins (Fig. 1). The spacing of cysteine residues in the two putative extracellular domains, $C(X_6)C(X_3)C$ (residues 54–65), and $C(X_4)C(X_5)C$ (residues 168–179), was identical to all other connexins, except Cx31. This new connexin was designated Cx30.2, or α11 connexin (GJA11), according to nomenclatures previously pro-

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M  G  E  W  A  F  L  G  S  L  L  D  P  L  Q  V  Q  S  P  L      20
ATGGGGGAGTGGGCGTTCCTAGGCTCCCTGCTGGACCCGCTGCAGGTACAGTCGCCGCTC   60
V  G  R  L  W  L  V  I  M  L  I  F  R  I  L  V  L  A  T  V      40
GTGGGTCGTCTCTGGCTGGTGATCATGCTGATCTTCCGCATCCTGGTGCTGGCCACGGTG  120
G  G  S  V  F  E  D  E  Q  E  E  F  V  C  N  T  L  Q  P  G      60
GGAGGTTCGGTGTTCGAGGACGAGCAGGAGGAGTTCGTGTGTAACACGTTGCAGCCCGGC  180
C  R  Q  T  C  Y  D  R  A  F  P  V  S  H  Y  R  F  W  L  F      80
TGTCGCCAGACCTGCTACGATCGCGCCTTCCCGGTGTCCCACTACCGCTTCTGGCTCTTC  240
H  I  L  L  L  S  A  P  P  V  L  F  V  I  Y  S  M  H  Q  A     100
CACATCCTGCTGCTGTCGGCGCCGCCGGTGCTGTTCGTCATCTACTCCATGCACCAGGCC  300
S  K  E  A  G  G  A  Q  L  A  P  P  C  A  R  G  R  A  E  A     120
AGCAAGGAGGCGGGTGGTGCGCAGCTGGCCCCGCCGTGCGCGCGCGGGCGTGCCGAGGCG  360
P  C  S  P  C  A  L  R  A  R  R  A  R  R  C  Y  L  L  S  V     140
CCGTGCTCCCCGTGCGCCCTGCGCGCTCGCCGCGCGCGCCGCTGCTACCTGCTGAGCGTG  420
A  L  R  L  L  A  E  L  A  F  L  G  G  Q  A  L  L  Y  G  F     160
GCTCTGCGCCTGCTCGCCGAGCTGGCTTTCCTGGGCGGCCAGGCGCTGCTCTACGGCTTC  480
R  V  D  P  H  Y  A  C  A  G  P  P  C  P  H  T  V  D  C  F     180
CGCGTGGACCCGCACTACGCGTGCGCCGGGCCACCTTGTCCGCACACGGTCGACTGTTTC  540
V  S  R  P  T  E  K  T  V  F  V  V  F  Y  F  A  V  G  L  L     200
GTGAGCCGGCCCACCGAGAAGACCGTCTTCGTGGTCTTCTACTTCGCCGTCGGGCTGCTG  600
S  A  L  L  S  V  A  E  L  G  H  L  L  W  K  G  R  Q  R  A     220
TCGGCGCTGCTCAGCGTGGCGGAGCTGGGTCACCTGCTCTGGAAGGGTCGCCAGCGCGCC  660
K  L  L  P  P  P  P  P  S  P  S  L  P  S  Q  R  G  D  P  D     240
AAGCTGCTCCCGCCGCCGCCGCCGTCGCCCTCTTTGCCATCGCAGCGCGGGGACCCCGAC  720
P  F  G  P  P  A  Y  A  H  R  S  P  A  G  D  S  E  G  E  G     260
CCTTTCGGCCCGCCAGCCTACGCGCACCGCTCACCGGCAGGCGACAGCGAGGGCGAAGGC  780
G  S  G  H  S  K  A  S  L  A  T  V  R  Q  D  L  A  I          278
GGCAGCGGCCACAGCAAAGCGTCGCTGGCTACCGTGCGCCAGGACCTGGCCATCTAGCGG  840
CGAGATCGGAGGCTGGATATCAGCACCCGGGTCCTTGGAGGCAGAAGGCTTTGCTTCTGA  900
AAGACTGCGTAGGACCCTGCCTGAGCAGAAGATGAGCAGAGGGGACCGCCAGGGTCGGG  960
TGGGGGAGGTGTCGCACTTCCTAGTGCTGGTTACGGCAGCCGTGTTAAATCAGGGAGGCT 1020
CCTCGGTGCTGTGATCCCTGCGGAAAGGGAGGACGCCAAGGGCTCCCCTGAGAGGCGGCA 1080
AACCGGCCGTGGGTGACCTGATTGTTGCACCGGGGACAAAAATCAACAGTGATGGCAACA 1140
TTCCCAGGTTCCCTGCTAATTCAGAGAAGAGTGGTTTTCCGTGAGCGCTCTGCACACACT 1200
CTCTCTGGACACACTCAGATGGGTTGGCTGCTCAAGCATAGTAAACCTCTATGAGCTGTG 1260
TGTATTTGCATGTGTATGCTTGTGAGGGTCCGCACATGCGTGTGTCCACACGTATACATC 1320
TCCCAAGTGACAGGCGATAGAGACCTGGGTTCATGCCTGCGTGCACAGAAATGTACATGT 1380
ATGTCCTTGAAGGCTCTGTGTGGATGCATGTACGCATGCGTCCTGTGTTAGCTCTGTATA 1440
CACACCTATACACACGTACATTCTGTGTGTCATCCGCCACCACTGTCAGTCACTAACCC 1500
AGCCACGTATGAGGCAACTCTGTCCCCAACAGGAACTGACAGCAAACAGTCCTGTTCAGT 1560
CAGATGTGTTCTGGGCCCAGTGGCCAGGCAGGGTAGCCCAGTGGCTAGGCAGGGTAGCTA 1620
AGCTCCACCTTGGAGGCTGACTGGGACTGGGGTGGGGCTCGACTTGAAACAGTGCTGTGC 1680
TTTCGGCTTACTTCATCTCTGGTCCCACCTGCCATTGTTCCCTGTAATCTATAAATGTGA 1740
CTGACTCCTACCAATCGTCAAGGAAAGCTGCCCTTCAAAGAGAGGTGTTTCTGGGTCCCC 1800
ATTCCCTTGCAACTAG                                              1816
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Fig. 1. Genomic locus containing the Cx30.2 open reading frame. This sequence, and surrounding genomic sequence represented in Fig. 3, has been deposited in the GenBank™ database under accession number AY166870. Putative transmembrane regions are underlined. Cysteine residues conserved between connexins are circled. The most 3′ bases contained in two different 3′RACE products are indicated in bold (nucleotides 1263 and 1816). A putative poly(A)$^+$ recognition sequence is underlined (nucleotides 1239–1244).

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mCx30.2 MGEWAFLGSLLDAVQLQSPLVGRLWLVIMLIFRILVLATVGGAVFEDEQEEFVCNTLQPG 60
hCx31.9 MGEWAFLGSLLDAVQLQSPLVGRLWLVVMLIFRILVLATVGGAVFEDEQEEFVCNTLQPG 60

mCx30.2 CRQTCYDRAFPVSHYRFWLFHILLLSAPPVLFVIYSMHQASKEAGGAQLAPPCARGRAEA 120
hCx31.9 CROTCYDRAFPVSHYRFWLFHILLLSAPPVLFVVYSMHRAGKEAGGAEAAAQCAPGLPEA 120

mCx30.2 PCSPCALRARRARRCYLLSVALRLLAELAFLGGQALLYGFRVDPHYACAGPPCPHTVDCF 180
hCx31.9 QCAPCALRARRARRCYLLSVALRLLAELTFLGGQALLYGFRVAPHFACAGPPCPHTVDCF 180

mCx30.2 VSRPTEKTVFVVFYFAVGLLSALLSVAELGHLLWKGRQRA---------------KLLPP 225
hCx31.9 VSRPTEKTVFVLFYFAVGLLSALLSVAELGHLLWKGRPRAGERDNRCNRAHEEAQKLLPP 240

mCx30.2 PPPSP---SLPSQRGDPDPFGPPAYAHRSPAGDSEGEGGSGHSKASLATVRQDLAI 278
hCx31.9 PPPPPPPPALPSRRPGPEPCAPPAYAHPAPA--SLRECGSGRGKASPATGRRDLAI 294
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Fig. 2. Amino acid sequence similarity shared between Cx30.2 and Cx31.9. Putative PKC and casein kinase II phosphorylation sites are indicated in bold italics. Putative transmembrane regions are boxed. Stars indicate conserved residues, double dots similar residues, and single dots like residues. Note the additional 15 amino acid residues present in Cx31.9.

posed for connexin genes [10,11]. Comparison between Cx30.2 and Cx31.9, using ClustalW, revealed that the proteins contained 79% identical residues, and 84% similar residues (Fig. 2). This similarity is consistent with Cx30.2 and Cx31.9 being orthologues, since the similarity between other connexin orthologues from human and mouse ranges between 50 and 99%. Also at the DNA sequence level, the Cx30.2 (834 bp) and Cx31.9 (882 bp) open reading frames showed 78% sequence identity, supporting that these are orthologous genes. Furthermore, an extremely high GC content was found in the coding region of *Cx30.2* (70%), similar to the GC content of *Cx31.9* (76%).

Interestingly, although the C-terminal cytoplasmic region is the domain with the least amino acid sequence similarity between Cx30.2 and Cx31.9 (56% identical residues), the most C-terminal four residues, DLAI, are completely conserved. We have shown that these residues are most likely involved in binding of Cx31.9 to the second PDZ domain of ZO-1 [5], and this may also be the case for Cx30.2.

A number of consensus motifs for potential posttranslational modifications were identified by searching the Cx30.2 and Cx31.9 sequences against the PROSITE database. In the C-terminal cytoplasmic domain, three putative phosphorylation sites were conserved between the mouse and human genes (numbers indicate amino acid residue numbers for Cx30.2 and Cx31.9, respectively). Two conserved potential protein kinase C (PKC; S/T-X-R/K) phosphorylation sites were identified at residues 234/252, and at residues 271/287. Cx31.9 contained two further putative PKC sites not present in Cx30.2, at residues 272 and 278. A potential casein kinase II site (S/T-X-X-D/E) was conserved at residues 256/272. Furthermore, a conserved putative casein kinase II site is present in the N-terminal region, at residues 9/9 (Fig. 2).

However, one striking difference between Cx31.9 and Cx30.2 is a 15 amino acid residue insertion in C-terminal region of Cx31.9, which is absent from Cx30.2. This insertion is located 58 residues from the C-terminus. Interestingly, human Cx46 also has a 15 amino acid insertion when compared with its mouse orthologue, at approximately the same distance from the C-terminus (64 residues). The sequences inserted in the human connexins do not share any significant similarity, except for a small motif, AHEEA in Cx31.9, and AHEAEA in Cx46. Whether these domains are involved in a common gain of function of Cx31.9 and human Cx46 compared to their mouse counterparts, e.g. the ability to interact with another protein or domain, only present in human, of if these insertions are coincidental, remains to be determined.

During the course of this study, Eiberger et al. have identified the sequence of the gene encoding Cx30.2 from the Celera database, and recently deposited this sequence in the GenBank database under accession number CAC93843 [3,4]. Furthermore, Belluardo et al. have recently reported the cloning of a mouse homologue of Cx31.9 with a molecular weight of 30 216, which presumably is identical to the gene reported here, although no sequence information has been provided [6]. The NCBI Annotation Project has recently deposited the sequence of Cx30.2 in GenBank with the accession number XP_137843. This sequence is derived from a genomic clone, which was localized to mouse chromosome 11. This is a region with conserved synteny to human chromosome 17q21, the localization of *Cx31.9*, further strengthening the assumption that these may be orthologous genes. Recently, human Cx45 (GJA7) has also been mapped to chromosome 17q21.31 by The Human Genome Sequencing Project (HUGO), and the mouse orthologue of Cx45 has been mapped to mouse chromosome 11 (Mouse Genome Informatics accession ID: MGI:95718). Thus, this syntenous chromosomal locus contains both the genes encoding Cx31.9/Cx30.2, and Cx45. The precise distance between these genes remains to be determined, but they do not appear to be in the immediate vicinity of each other.

The genomic sequence upstream of the human Cx31.9 gene has been partly sequenced by us, and further sequence can be found from a genomic clone with GenBank accession number AC090426.1. This sequence was compared with the genomic sequence upstream of the Cx30.2 open reading frame. The first 13 bp upstream of the initiating ATG codon are completely conserved, except position −2, which is T in mouse and C in human. This base substitution does not interfere with optimal translation [12]. Further upstream, the nucleotide similarity decreases rapidly.

In order to obtain sequence of the 3′UTR of the Cx30.2 gene, 3′RACE was performed using commercial mouse cDNA. This experiment suggested that at least two different 3′UTRs were present in mouse brain, one of 427 bp, and one of 980 bp (excluding the poly(A)$^+$ tail) (Fig. 1). For the shortest 3′RACE product, the sequence AGTAAA was found 24 nucleotides upstream of the poly(A)$^+$ site. Although this sequence is not identical to the normal AAUAAA poly(A)$^+$ recognition sequence, it could possibly serve as such. No po-

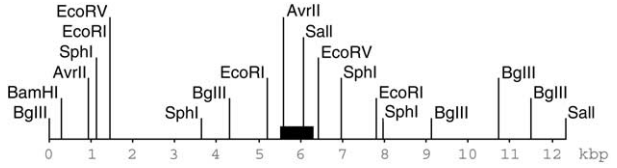

Fig. 3. Restriction map of the genomic locus (12 374 bp) containing the Cx30.2 open reading frame (indicated by a thick line).

tential poly(A)$^+$ recognition sequence was observed for the long 3′RACE product. This sequence could be an artifact, since the oligo-dT primer used to prime cDNA synthesis may occasionally anneal to sites other than the poly(A)$^+$ tail. Further studies are needed to clarify this issue.

The 3′RACE fragment was used to identify a genomic 5930 bp *Eco*RV/*Sal*I fragment comprising sequence downstream of the Cx30.2 open reading frame. Alignment of the genomic sequence and 3′RACE products revealed that the genomic sequence was uninterrupted by introns, as is the case for the 3′UTR regions of all connexin genes characterized to date.

A genomic fragment further upstream than the 4981 kb *Eco*RV fragment was also subcloned from the λ genomic clone. In total, 12 374 bp containing and surrounding Cx30.2 were sequenced, and have been deposited in the GenBank/EMBL database under accession number AY166870 (Fig. 3).

3.2. Northern analysis of Cx30.2 expression

In order to examine whether a transcript originated from the *Cx30.2* gene, a probe from the 3′ end of the Cx30.2 coding region, which showed no similarity to other connexin genes, was used to probe a Northern blot of adult mouse tissues. A signal was detected in all tissues tested, as was the case for human Cx31.9. The signal from testis was several fold stronger than the second most abundant signal, which was from heart. In contrast to human Cx31.9, which only had one major transcript migrating at 4.4 kb, several transcript sizes were detected for Cx30.2, at 3.5, 2.2, 1.6, and 1.2 kb. Not all these transcripts were detected in every tissue (Fig. 4). This pattern was unusual, since most connexin genes only give rise to a single transcript size, although some genes, such as Cx30, Cx30.3, and Cx31, show two transcript sizes. To examine the specificity of the probe for the Cx30.2 sequence, the same probe was used in Southern blot analysis of mouse genomic DNA under the same stringency. In this experiment, the probe only hybridized to the expected 12 kb *Bam*HI genomic fragment (Fig. 4B), indicating that it is specific for the Cx30.2 sequence.

The exact mechanism by which the different transcripts are generated remains unclear, but alternative usage of different poly(A)$^+$ sites, which was indicated by the 3′RACE experiments (Fig. 1), may be a partial explanation.

3.3. Generation of antibodies against Cx30.2 and immunoblot analysis

Polyclonal antibodies were raised against the Cx30.2 protein. Since it is known that anti-connexin antibodies in some instances cross react with other connexins, we decided to raise two different antibodies against Cx30.2. To further reduce the risk of cross reactivity, we chose to generate the antibodies against domains that show the least similarity to other connexins, namely the cytoplasmic loop (L) and the C-terminal domain (T). These respective domains were cloned into a prokaryotic expression vector and expressed in bacteria as GST fusion proteins. Upon purification, these fusion proteins were observed to migrate more slowly in SDS–PAGE as compared to the migration of GST, due to the added mass of the connexin domains (not shown). pAbs were raised against the L and T Cx30.2 fusion proteins, affinity purified, and cross absorbed on columns containing GST alone. The resultant antibodies were used to probe immunoblots of lysates from HEK cells overexpressing Cx30.2 and mouse tissues. The Cx30.2-T pAb detected a band migrating around 30 kDa in Cx30.2 transiently overexpressing cells (Fig. 5, lane 1), but not from wild type (wt) cells (lane 2), or cells transiently overexpressing Cx43 (not shown), indicating that the band represented Cx30.2. A number of mouse tissue lysates were also tested. A band migrating around 30 kDa was detected in testis lysates, but not in any of the other tissues tested despite long exposure (lane 3, arrow). The protein detected from testis migrated as a more compact band compared with Cx30.2 overexpressed cell culture. This may be due to differences in phosphorylation or other posttranslational modifications of Cx30.2, and/or differences in abundance. Ponceau staining of the membrane (not shown), and reprobing of the blot with an antibody against actin, indicated that comparable amounts of protein were loaded from all mouse tissues (Fig. 5, middle panel). Lesser quantities of total protein were loaded from the cell lysates, to avoid overexposure due to the intensity of the Cx30.2 signal from transfected cells. An intensely labeled protein was also detected in brain migrating around 80 kDa (lane 5, arrowhead). Detection of this protein was most likely due to cross reactivity with the T pAb, due to an epitope shared between this unknown protein and Cx30.2. The cross reactive protein was only detected in large amounts in brain, although liver showed weak bands in the same area of the blot upon longer exposure (not shown). The presence of this putative cross reactive protein suggested that the T pAb is not useful for IF studies of mouse brain.

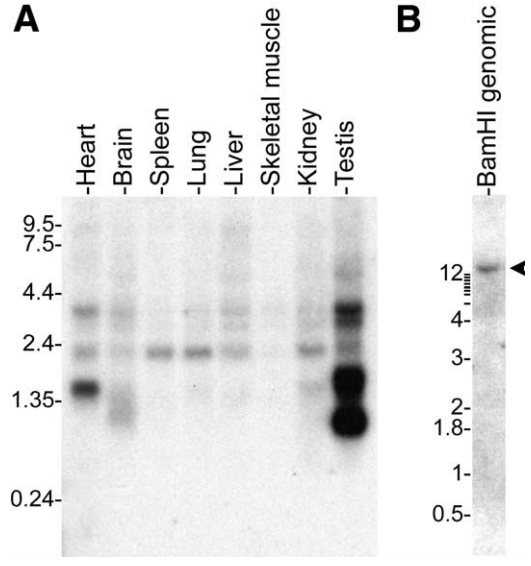

Fig. 4. Expression pattern of Cx30.2. A: Northern analysis of Cx30.2 transcripts using a probe from the 3′UTR. Transcripts of 3.5, 2.2, 1.6, and 1.2 kb were detected, most prominently in testis. B: The same probe was used to probe a Southern blot containing 129/Sv *Bam*HI digested DNA. A single band migrating above 12 kb is seen, confirming the specificity of the probe.

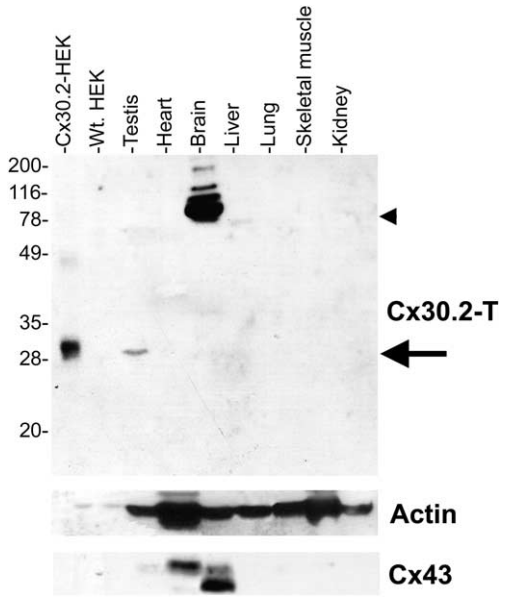

Fig. 5. Immunoblot using the Cx30.2-T pAb (upper panel) on Cx30.2 overexpressing HEK293 cells, wt HEK293 cells, and mouse tissue lysates. A protein migrating at approx. 30 kDa was detected in Cx30.2 overexpressing cells, and from mouse testis (arrow). A cross reactive protein migrating at 80 kDa was observed only in brain lysates (arrowhead). Reprobing of the blot for actin showed the amounts of protein loaded from each tissue. Considerably less protein was loaded from the HEK cell lysates, due to the intensity of the Cx30.2 signal in the transfected cells. Probing of an identical blot for Cx43 showed the amounts of this connexin in the tissue lysates.

Based on the results of the Northern analysis, it was not surprising that the highest expression level of Cx30.2 was found is testis by immunoblot analysis. However, the Northern analysis indicated that Cx30.2 was expressed in all tissues tested. The lack of Cx30.2 protein detected in tissues other than testis was most likely due to low expression levels in other tissues, or to a general low level of connexins in the total tissue lysates analyzed. To visualize the levels of connexins in the lysates, an identical blot was used to detect Cx43 (Fig. 5, lower panel). Cx43, which is abundantly expressed, could only be detected in heart and brain, with very weak signals from testis and skeletal muscle. Thus, the lysates likely contained low amounts of connexins, and presumably special extraction procedures, such as enriching for membrane fractions, will increase the detection limits, and may be needed to detect Cx30.2 in tissues other then testis.

The Cx30.2 L pAb was not able to detect overexpressed Cx30.2 from HEK cells by immunoblot analysis, despite using large amounts of antibody and tissue lysate. This pAb also did not detect Cx30.2 from any of the mouse tissues tested in immunoblot analysis. Interestingly, the mAb we previously generated against the cytoplasmic loop region of human Cx31.9, mAb 5G11, also did not work in immunoblot analyses, although it worked well in immunoprecipitation and by IF [5]. Possibly, the immunogenic epitopes of this domain in Cx30.2, and in Cx31.9, are destroyed by SDS.

3.4. Detection of Cx30.2 in overexpressing cells and mouse tissues

The Cx30.2 T and L pAbs were then used in IF and confocal microscopy of Cx30.2 transiently transfected HEK cells, and wt HEK cells. With both pAbs, about 10% of the cells transfected with Cx30.2 were intensely labeled, corresponding to the transfection efficiency in the experiment shown (Fig. 6A,C,D). Both pAbs thus recognized Cx30.2 by IF. In contrast, wt cells (Fig. 6B,E), or Cx43 transfected cells (not shown) were not labeled by either pAb. Between some Cx30.2 expressing cell pairs, structures resembling gap junctions were observed, both with the Cx30.2 L pAb (Fig. 6C, arrow), and with the Cx30.2 T pAb (Fig. 6D, arrows). Furthermore, Cx30.2 was found to form similar structures between cells when HEK cells were transfected with a different construct, which contained an eGFP tag attached to the C-terminus of the connexin (Fig. 6F, arrows). These results indicate that Cx30.2 has the ability to traffic to the plasma membrane in areas of cell–cell contact, where it most likely forms gap junctions.

Analyses of the tissue and cell specific expression of Cx30.2 in adult mouse tissues using the Cx30.2 T and L pAbs showed

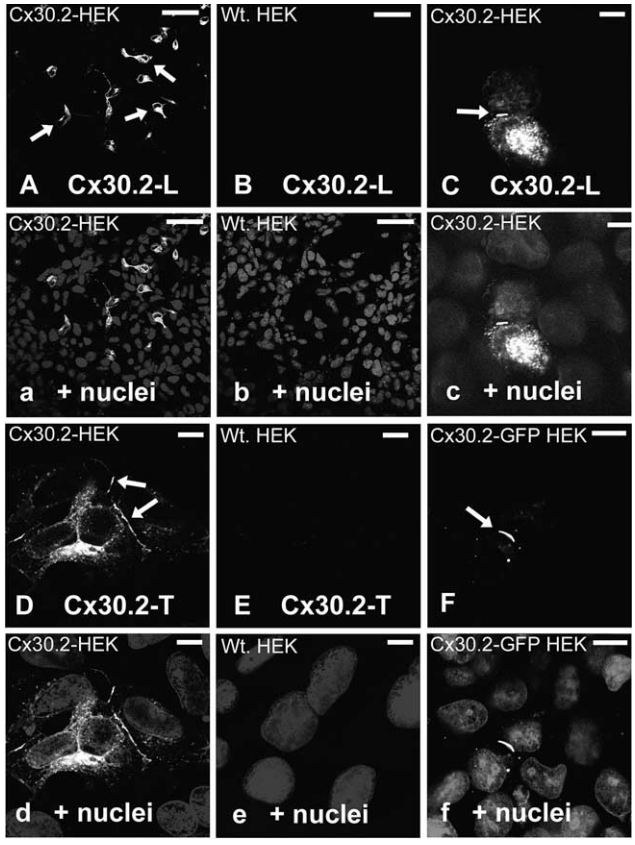

Fig. 6. Confocal IF microscopy of Cx30.2 transfected cells. The Cx30.2 L and T pAbs recognize Cx30.2 transfected HEK cells, and Cx30.2 traffics to the cell surface, where it forms structures resembling gap junctions. GFP tagged Cx30.2 also traffics to the cell surface. Confocal IF microscopy showing HEK cells transiently transfected with Cx30.2 (A,C,D), untransfected HEK cells (B,E), or fluorescence of Cx30.2-GFP transiently transfected HEK cells (F). Fluorescence only is shown in rows 1 and 3, while fluorescence combined with a nuclear stain is shown in rows 2 and 4 (marked a–f). The Cx30.2-L pAb is seen to stain Cx30.2 expressing cells (A, arrows), but not wt HEK cells (B). Between some transfected cell pairs, gap junction like structures were seen between cells (C, arrow). Similarly, the Cx30.2 T pAb stains gap junction like structures between transfected cells (D, arrows), but not wt cells (E). Similar structures were also observed between some cell pairs overexpressing Cx30.2-GFP (F, arrow), but not GFP alone (not shown). Scale bars: A,B: 100 μm; C–F: 10 μm.

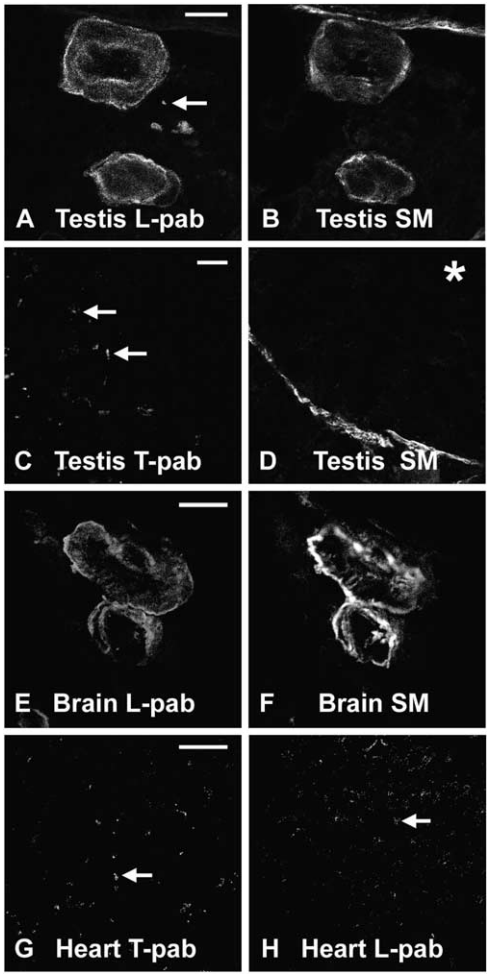

Fig. 7. Detection of Cx30.2 in mouse tissues by IF and confocal microscopy. Adult mouse tissues were stained with Cx30.2 T and L pAbs, and with a marker for smooth muscle cells (SM). Panels A–B, C–D, and E–F are double stainings with the channel containing the Cx30.2 pAb to the left, and the SM channel to the right. A,B: In testis, Cx30.2 was expressed in smooth muscle cells, but also in interstitial cells (arrow). C,D: In seminiferous tubules, a fine punctate, or fine line, staining was observed, possibly between Sertoli and germ cells (arrows). The asterisk in D marks the lumen of the tubule, and the smooth muscle staining is lymphatic vessels encircling the tubule. E,F: In brain, smooth muscle in blood vessels was stained by the L-pAb. G,H: In heart, both Cx30.2 pAbs labeled intercalated discs. Scale bars: A–F: 10 μm; G,H: 100 μm.

that a similar staining pattern was generally obtained with both pAbs. In testis, staining of blood vessels was observed (Fig. 7A). Double labeling with a mAb against smooth muscle cells, revealed that these were labeled by the Cx30.2 pAbs (Fig. 7B). However, staining of structures in the interstitial cells was also observed with both pAbs (Fig. 7A, arrow). Furthermore, staining within the tubules, possibly between Sertoli and germ cells, was also observed (Fig. 7C, arrow). The lymphatic smooth muscle cells surrounding the seminiferous tubules (Fig. 7D) were not significantly stained by either Cx30.2 pAb, although occasionally a low level of staining was observed. Thus, when compared to our findings regarding Cx31.9 expression in human testis, which were limited to smooth muscles in blood vessels, Cx30.2 seems to be more widely expressed, both in intestinal cells and in cells of the seminiferous tubules. In brain, the Cx30.2-T pAb stained a majority of cells intensely (not shown). This staining was probably due to the presumed cross reactivity observed by immunoblot analysis (Fig. 5). The L pAb stained vascular smooth muscle cells in brain (Fig. 7E,F). Furthermore, both the Cx30.2 T and L pAbs stained vascular smooth muscle cells in skeletal muscle (not shown). In heart, both pAbs stained intercalated discs (Fig. 7G,H, arrows).

Thus, despite some differences in the cell types expressing Cx30.2 and Cx31.9 in mouse and human, as well as differences in the number and size of the transcripts, we find that both connexins are expressed by vascular smooth muscle cells, that their amino acid sequences are highly similar, and that the Cx30.2 and Cx31.9 genes localize to syntenous chromosomal loci. These genes are thus most likely orthologues.

To investigate the biological function of Cx30.2, we are currently generating a mouse homozygous for a targeted deletion of this gene.

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