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A simple RT-PCR-based strategy for screening connexin identity

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Abstract

Vertebrate gap junctions are aggregates of transmembrane channels which are composed of connexin (Cx) proteins encoded by at least fourteen distinct genes in mammals. Since the same Cx type can be expressed in different tissues and more than one Cx type can be expressed by the same cell, the thorough identification of which connexin is in which cell type and how connexin expression changes after experimental manipulation has become quite laborious. Here we describe an efficient, rapid and simple method by which connexin type(s) can be identified in mammalian tissue and cultured cells using endonuclease cleavage of RT-PCR products generated from "multi primers" (sense primer, degenerate oligonucleotide corresponding to a region of the first extracellular domain; antisense primer, degenerate oligonucleotide complementary to the second extracellular domain) that amplify the cytoplasmic loop regions of all known connexins except Cx36. In addition, we provide sequence information on RT-PCR primers used in our laboratory to screen individual connexins and predictions of extension of the "multi primer" method to several human connexins.

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- Gap junctions
- Intercellular communication
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- Multi primers

Introduction

Gap junction channels are found in most tissues where they provide conduits for diffusion of ions and small molecules between neighboring cells. Cloning studies have thus far revealed fourteen distinct connexin (Cx) cDNA sequences encoded by different genes in rodent tissues with highly homologous isoforms in other mammals. In mammals, group I (ß) connexins include Cx26, Cx30, Cx30.3, Cx31, Cx31.1 and Cx32, and group II (α) connexins include Cx33, Cx37, Cx40, Cx43, Cx45, Cx46 and Cx50 (1). In addition, a new connexin type (named Cx36) has been identified in the mammalian brain and assigned to a new group of connexins termed group III (γ) (2,3).

Methods that have become standard for establishing identities of connexins within specific tissues include Northern blot and Western blotting and immunocytochemical assays. However, these methods require relatively high amounts of RNA and protein, and detection of protein requires connexin-specific antisera (few of which are commer-

cially available). To circumvent these drawbacks, we have developed a rapid method for screening connexin identity using RT-PCR techniques. Because nucleotide sequences of connexin cDNAs are highly conserved in both intramembrane and extracellular domains, a pair of degenerate oligonucleotide primers were previously designed to amplify the cytoplasmic loop regions of all known connexins (4). Here, we have utilized this pair of "multi primers" to amplify connexin DNA sequences starting from total RNA and identified individual connexin species through the use of specific endonucleases; amplification of the cytoplasmic loop regions results in major bands that distinguish group I from group II connexins. We show that group I connexins display sizes on gels of 350-390 bp, while group II connexin sequences amplified by these primers are 420-520 bp. We also show that certain connexins are not readily identified by this technique and propose connexin-specific RT-PCR primers to overcome this limitation.

In the present study we amplified cloned cDNAs representing connexins from groups I and II (Figures 1 and 2) and identified connexins generated by reverse-transcribed RNA samples from a neural cell line (Figure 3); the identity of the connexin was confirmed by sequencing. Finally, we demonstrate the applicability of our method by detecting the connexins expressed in the liver (i.e., Cx26, Cx32 and Cx43; Figure 4 and Table 1). We provide a detailed "bench-top" protocol and a succinct description of our methodology, discuss its potential applica-

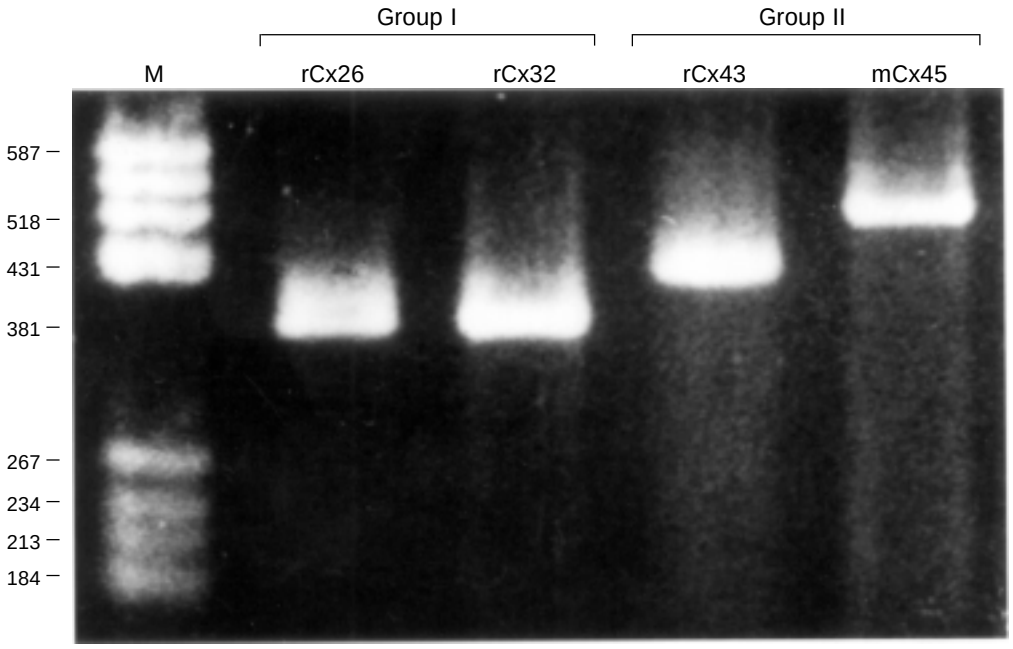

Figure 1 - Detection of connexins belonging to groups I (rCx26, rCx32) and II (rCx43, mCx45) using "multi primers" with connexin-cloned cDNA. PCR products corresponding to 382 bp for Cx26, 381 bp for Cx32, 431 bp for Cx43, 518 bp for Cx45. Molecular markers are indicated on the left (M). r, Rat; m, mouse.

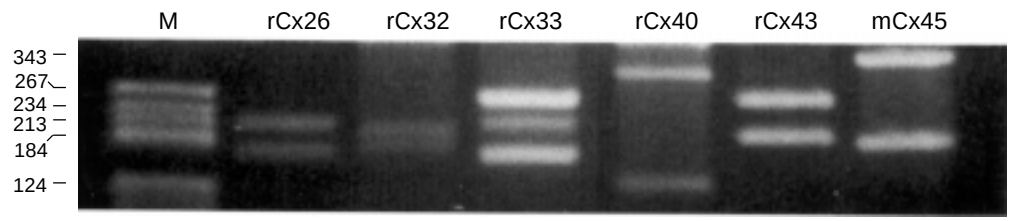

Figure 2 - Digestion of PCR products of clonned connexin cDNAs by specific endonucleases. The endonucleases used were *Mse*I (rCx26), *Eco*NI (rCx32), *Mse*I (rCx33), *Fsp*I (rCx40), *Hin*cII (rCx43), and *Eco*RI (mCx45). Molecular markers are indicated on the left (M). r, Rat; m, mouse.

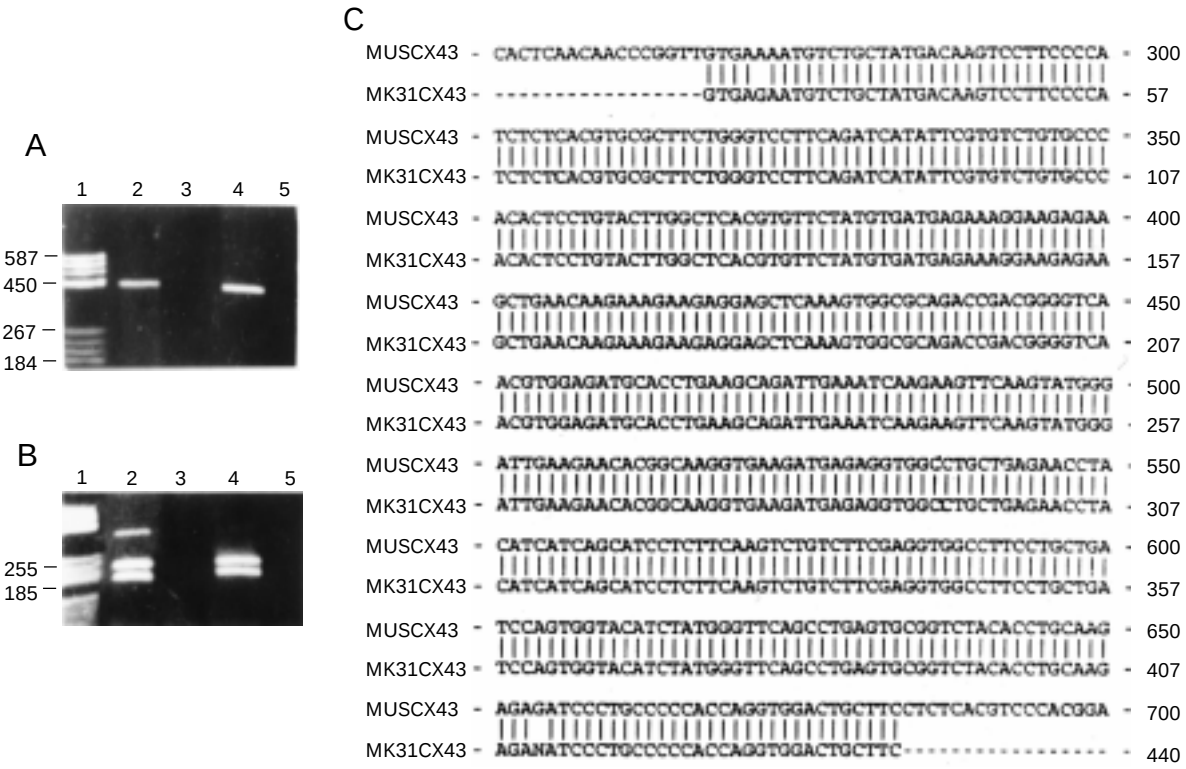

Figure 3 - Connexin detection in a neural cell line (MK31 cell line). Bench-top protocol of our methodology and potential applications. *A*, Screening connexin identity in a cell line using RT-PCR assays. First generation of PCR products indicates that the amplified DNA sequences belong to group II (A2,4). *B2*, Second generation of PCR products indicates the expression of Cx43 in these cells. Lane 4 is the PCR product of Cx43 from rat heart and lane 5 is its corresponding negative control. *B*, Lanes 2,4 show the presence of restriction products of Cx43 after *Hin*cII was used. Lane 3 is a negative control for the cells. The negative controls were run in PCR without reverse transcriptase to verify that genomic DNA was not present (lanes 3,5). *C*, Sequencing the RT-PCR products revealed the presence of Cx43 (99.7% identical) in these cells. Terminology: Mouse heart Cx43 sequence (MUSCX43) and sequence found in the neural cell line (MK31CX43). Molecular markers (A1, B1).

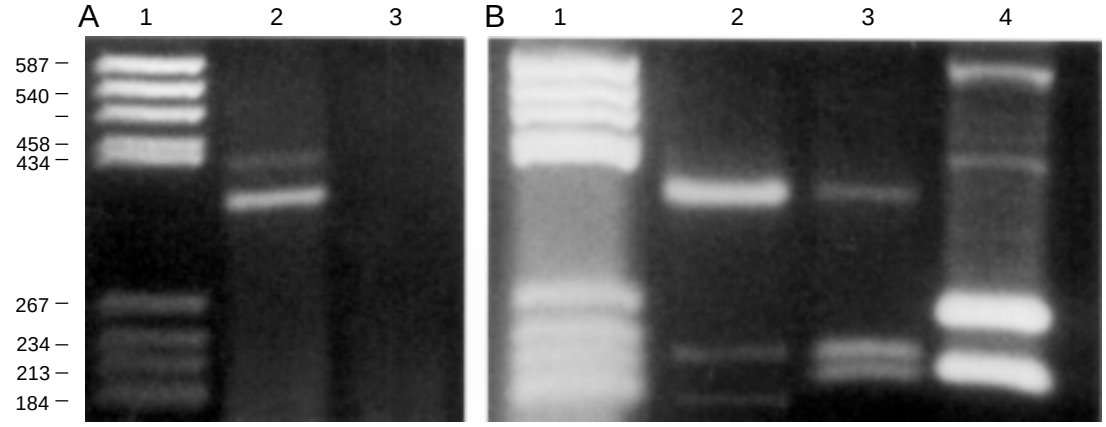

Figure 4 - Determination of the connexin types expressed in rat liver. *A*, First generation of PCR products indicates that the amplified DNA sequences belong to groups I and II (lane 2), as expected, and its corresponding negative control (lane 3). Molecular markers (lane 1). *B*, Second generation of PCR products. For this figure, first round PCR bands were isolated from the gel and PCR products belonging to Cx26 (lane 2), Cx32 (lane 3) and Cx43 (lane 4) were identified, as expected. Molecular markers (lane 1). The endonucleases used were *Mse*I (Cx26; lane 2), *Eco*NI (Cx32; lane 3) and *Hin*cII (Cx43; lane 4), respectively.

tions and limitations and provide advice on avoiding potential pitfalls. In addition, we also provide GenBank access numbers for connexin types expressed in rodents and humans (Tables 1 and 2), a list of specific restriction enzymes to be used and sequences for connexin-specific primers (Table 3) routinely used in our laboratory. Thus, this communication provides a starting point for those new to the gap junction field and should also serve as a useful reference for more experienced researchers.

Material and Methods

Preparation and treatment of RNA

Total RNA was isolated from cultured cells or tissues by a procedure based on that of Chomczynski and Sacchi (5), quantitated by absorbance measurements at 260 nm and 280 nm (Hitachi U-1100) with 1 OD unit considered equal to 40 µg/ml. Ten U of DNase I (Boehringer Mannheim, Indianapolis, IN, USA) was added to each 5 µg of RNA and incubated at 25°C for 15 min to digest residual genomic DNA. DNase was heat-inactivated at 65°C for 15 min before the RT-PCR analyses.

RT-PCR analyses

First strand cDNA was synthesized from total RNA templates using random primers and the superscript preamplification system (Gibco BRL-Life Technologies, Inc., Grand Island, NY, USA) (6). One to two micrograms of total RNA was brought to 11 µl in diethyl pyrocarbonate (DEPC)-treated water and combined with 1 µl random hexamers (50 ng/µl). The mixture was heated at 70°C for 10 min and then incubated on ice. The remaining components for reverse transcription were then added and incubated for 10 min as follows: 2 µl of 10x synthesis buffer, 2 µl 0.1 M DTT, 2 µl 25 mM $MgCl_2$, 1 µl 10 mM dNTP mix and 1 µl (200 units) superscript reverse transcriptase. The reaction mix was left at room temperature (RT) for 10 min, incubated at 42°C for 50 min and the reaction terminated by incubating at 70°C for 15 min.

Degenerate oligonucleotides corresponding to conserved regions of the first and second extracellular connexin domains were synthesized on an ABI model 391 Sequencer (Applied Biosystems, Foster City, CA, USA). The sequences of the 24-mer sense and 21-mer antisense "multi primers" (4) are given below (using IUB group codes):

A) Sense 5' GGC TGT RAV AAY GTC TGC TAY GAC 3'

B) Antisense 5' TGG GVC KGG AVA BGA AGC AGT 3'

PCR reactions contained (in a final volume of 50 µl): 5 µl of RT reaction (0.5-1 µg of first strand cDNA), 1 µM of sense and antisense primers, 5 µl of 10x PCR buffer (100 mM Tris-HCl; 500 mM KCl, pH 8.3), 0.2 mM dNTP, 3 mM $MgCl_2$ and 2.5 U Taq polymerase (Gibco BRL-Life Technologies, Inc.). Thirty cycles were performed on the samples using a PTC-100 Thermocycler (M.J. Research Inc., Watertown, MA, USA) as follows: 1) denaturation at 94°C for 30 s; 2) annealing at 55°C for 30 s; 3) extension at 72°C for 30 s. This was followed by a final extension cycle at 72°C for 8 min and a soak cycle at 4°C. Reaction products were analyzed by electrophoresis on 2% agarose gels in order to detect contaminants. The corresponding bands were isolated from gels and purified (Qiagen Inc., Valencia, CA, USA). The DNA was reamplified, and restriction digestion analysis with specific enzymes (New England Biolabs Inc., Beverly, MA, USA) was performed to identify the connexins expressed in the rat liver and in a mouse neural cell line (MK31 cell line; 7,8).

Amplicon sizes were determined in agarose DNA gels following electrophoresis with markers corresponding to 8 to 587 bp.

DNA sequencing and analysis

RT-PCR products detected in hippocampal cells were sequenced and compared to murine cytoplasmic loop connexin sequences available at GenBank (Figure 3C).

Results and Discussion

This report describes an improved technique for the screening of tissue- or cell-specific RNA samples for the presence of mRNAs encoding members of the connexin multigene family. Previous studies have utilized RT-PCR techniques to detect connexin expression in different preparations (9-17). However, our methodology is novel in the ability to detect all known connexins belonging to groups I and II through the use of "multi primers" with only a single PCR reaction followed by specific restriction digestions. The previous methods required the use of specific conditions and specific primers for each connexin type and the reported methods lacked detailed experimental protocols. In addition, our study presents theoretical cleavage patterns of connexin PCR products specific for each connexin type as well as the GenBank access numbers for each connexin.

In contrast to previous publications, we have used the terminology "multi primers" instead of "universal primers", due to the fact that these degenerate oligonucleotides do not amplify the cytoplasmic loop regions of Cx36 (group III) and do not detect Cx37, Cx40 and Cx45 in a background where Cx43 is present.

The protocol we developed evolved from the use of degenerate oligonucleotides for PCR amplification of connexins reported by Haefliger et al. (4). Optimization of our preparation was achieved by reducing the duration of denaturation at 94°C from 1 min to 30 s, annealing at 55°C and extension at 72°C for 30 s and the final extension to 8 min. By reducing the duration of the cycles used by Haefliger et al. (4) by 50% (from 1 min to 30 s) and by increasing the concentration of $MgCl_2$ from 1.5 to 3 mM we have detected 8 connexin types in various cell and tissue preparations (Figures 1, 2, 3A,B, 4; Table 1).

"Multi primers" amplified sequences conserved among connexins, resulting in major bands that distinguished group I (350-390 bp) from group II (420-520 bp) connexins; these results are illustrated in Figure 1 for the group I connexins with Cx26 and Cx32 and for the group II connexins Cx43 and Cx45. Unique restriction sites within the amplicons generated by "multi primers" for each rodent and human connexin for which sequence information exists were deduced from the program PCGene (IntelliGenetics, Inc., Campbell, CA, USA). These predicted fragment sizes for rodent and human connexins are presented in Tables 1 and 2. Our predictions for the sizes of PCR product fragments after endonuclease treatment were confirmed using amplicons obtained from PCR with "multi primers" using cloned cDNAs encoding Cx26, Cx32, Cx33, Cx40, Cx43 and Cx45 (Figure 2); so far the only anomaly has been the presence of a canonical site for EcoNI in rat Cx32 DNA leading to an unexpected cleavage product of 213 bp (see Figure 2, rCx32).

In various cell lines and tissues, we have used this technique to detect Cx26, Cx32, Cx33, Cx36, Cx37, Cx40, Cx43, Cx46 and Cx50. Use of the method for detection of Cx43 in a neural cell line (MK31 cell line) is illustrated in Figure 3. We first assigned products as corresponding to group I or group II connexins on the basis of length (Figure 3A), and verified identity of the amplicons using digestion with endonucleases for unique sites (Figure 3B). Subcloning of these products and subsequent sequencing provided additional confirmation of the identity of the specific connexins expressed (Figure 3C). Figure 4 illustrates the applicability of this method to detect typical connexins expressed in the liver, i.e., Cx26 (Figure 4B2), Cx32 (Figure 4B3)

Table 1 - PCR products obtained from the 14 connexins expressed in rodents. Detection of connexin type identity from PCR products. PCR products of cDNA of Cx26, Cx30.3, Cx31, Cx31.1, Cx32, Cx33, Cx37, Cx40, Cx43, Cx45, Cx46 and Cx50.

r, Rat; m, mouse. Tissues with high expression, references for molecular cloning and GenBank database accession numbers are listed below. *A cleavage product of 213 bp, corresponding to a canonical site for *Eco*NI, is routinely found in rat liver. N.A., Not available; N.T., not tested.

Connexin (Cx)	GenBank access #	References	Restriction enzymes	Products (bp)
rCx26	X51615 (liver)	18	*Mse*I	169, 213
mCx26	M81445 (liver)	19	*Mse*I	170, 213
rCx30	N.A.	–	–	–
mCx30	Z70023 (skin)	20	*Fsp*I	177, 204
rCx30.3	X76168 (skin)	21	*Nr*UI	118, 248
mCx30.3	M91443 (skin)	22	*Sap*I	115, 252
rCx31	M59936 (skin)	23	*Ava*II	158, 211
mCx31	X63099 (skin)	24	*Ava*II	159, 211
rCx31.1	M76533 (skin)	4	*Xho*I	124, 236
mCx31.1	M91442 (skin)	22	*Xho*I	124, 237
rCx32	X04070 (liver)	25	*Eco*NI	178, 201*
mCx32	M81447 (liver)	19	*Eco*NI	177, 201*
rCx33	M76534 (testis)	4	*Mse*I	170, 265
mCx33	N.A.	–	–	–
rCx36	Y16898 (retina)	3	N.T.	N.T.
mCx36	AF016190 (brain)	2	N.T.	N.T.
rCx37	M76532 (lung)	4	*Bgl*II	204, 229
mCx37	X57971 (lung)	26	*Bgl*II	203, 230
rCx40	M76535 (lung)	4	*Fsp*I	124, 301
mCx40	X61675 (lung)	27	*Fsp*I	124, 303
rCx43	X06656 (heart)	28	*Hin*cII	185, 255
mCx43	X61576 (heart)	29	*Hin*cII	186, 247
rCx45	N.A.	–	–	–
mCx45	X63100 (heart)	24	*Eco*RI	177, 343
rCx46	X57970 (lens)	30	*Eag*I	185, 246
mCx46	N.A.	–	–	–
rCx50	N.A.	–	–	–
mCx50	M91243 (lens)	31	*Eco*NI	117, 324

Table 2 - Predicted unique cleavage sites for human gap junction proteins deduced from cDNA sequences.

References for GenBank database accession numbers, molecular cloning, restriction enzymes and predicted cleavage patterns.

Connexin (Cx)	GenBank access #	References	Restriction enzymes	Products (bp)
Cx26	M86849	32	*Mse*I	170, 213
Cx32	X04325	33	*Dde*I	100, 279
Cx37	M96789	34	*Bgl*II	203, 229
Cx40	U03486	35	*Dra*III	119, 307
Cx43	M65188	36	*Scr*FI	99, 333
Cx45	U03493	35	*Dde*I	197, 304

and Cx43 (Figure 4B4) (Table 1).

Precautions

Problems with RT-PCR sensitivity generally are associated with duration and temperature of incubation, numbers of cycles and reagents (in particular, Mg^{2+}). Incubation times should be kept as short as possible in order to optimize the protocol and minimize the occurrence of non-specific amplification. Since one of the key variables in this protocol is the concentration of Mg^{2+} and Mg^{2+} concentration requirements can vary dramatically depending on the sample to be studied, we recommend that a concentration curve for Mg^{2+} be established before completely implementing this protocol in order to avoid nonspecific priming and suboptimal enzymatic activity. For preliminary characterization of the connexins expressed in a specific tissue, we suggest the use of the "Q-solution" (Qiagen Inc.) which acts similarly to DMSO by modifying the melting behavior of DNA. More importantly, it has been shown that the concentration of Mg^{2+} does not need to be previously adjusted, providing an efficient amplification at a uniform concentration.

Although this method is now optimized to the point where it is reliable in discriminating many different connexin types, we have thus far been unable to detect Cx37, Cx40 and Cx45 in tissues where Cx43 is co-expressed. Nevertheless, one cannot rely only on the electrophoretic separation for identification of amplicons in agarose DNA gels. To substantiate the identity of the connexins, we strongly recommend one of the following strategies: i) all amplicons must be sequenced (as illustrated in Figure 3C), or ii) use of specific primers (Table 3), as still required for Cx37, Cx40, Cx45 and Cx36 (see 13,17). In our laboratory, immunocytochemical and functional electrophysiological assays have been routinely carried out in parallel to further substantiate the results obtained using the RT-PCR strategy.

Table 3 - Connexin-specific primers routinely used to verify the expression of connexins expressed in mice (*except Cx33 and Cx46).

*Mouse Cx33 and Cx46 sequences are not yet available (see Table 1); Cx33- and Cx46-specific primers are listed for rat sequences (e.g., rCx33 and rCx46).

Cx	Sequence (5'-3') (sense)	Sequence (5'-3') (antisense)
Cx26	AGATGGAGGGAGAGGATGAG	TCAGAGGAAGAGAAACAATGTG (312 bp)
Cx30	GGCTTGGTTTTCAGAGATAG	GAGTTGTGTTACCTGCTGC (369 bp)
Cx30.3	CACCGTATGTGATCTCCAAAG	ATGTTTCCCACCTGACCTG (199 bp)
Cx31	TGAAAGAAAGGAGATGGG	GCTTTTAAGGAAACGGAC (364 bp)
Cx31.1	CAAAGAATGACCCAAACTG	GACACCTTGAATCTGTAAACC (489 bp)
Cx32	TCCATCAAACCTTCCCTC	TTCTCTCTCCATAACTCCCTC (391 bp)
rCx33	AAACCATCTTCATCCTCTTC	GCTTTTCTGTCTACCTAAAACC (386 bp)
Cx36	GAGCAAACGAGAAGATAAGAAG	CCGCTTCTACATCATCCA (195 bp)
Cx37	GGCTGGACCATGGAGCCGGT	TTTCGGCCACCCTGGGGAGC (421 bp)
Cx40	TTTGGCAAGTCACGGCAGGG	TTGTCACTGTGGTAGCCCTGAGG (311 bp)
Cx43	TACCACGCCACCACTGGC	AATCTCCAGGTCATCAGG (407 bp)
Cx45	AAAGAGCAGAGCCAACCAAA	GTCCCAAACCCTAAGTGAAGC (313 bp)
rCx46	GGAAAGGCCACAGGGTTTCCTGG	GGGTCCAGGAGGACCAACGG (331 bp)
Cx50	GGAAGGAGGATGAGAAAG	GAGAATGGAGGAGGAAAG (462 bp)

Differences among connexin isoforms in mouse, rat and humans

Connexin sequences are found to be more conserved when comparing one isoform in different species (e.g., human and mouse Cx43 differ by only 12% at the nucleotide level and 2.5% at the amino acid level) than when comparing different isoforms in the same species (e.g., mouse Cx40 and Cx43 differ by 40% at the nucleotide level). Thus, the unique sites for endonucleases and the restriction fragment lengths might be conserved from mice to humans. However, evolutionary rates vary for different connexin genes among species (1). In rodents, the most striking difference that we have observed is related to rat/mouse Cx30.3 (Table 1). In humans, several connexins have also been cloned; their GenBank access numbers and predicted cleavage patterns are provided in Table 2. Note that Cx26 and Cx37 express endonuclease sites (and lengths of products) similar to those present in rat/mouse Cx26 and mouse Cx37, respectively.

Conclusions

In summary, the RT-PCR assay followed by connexin-specific endonucleases is simple, sensitive and reliable. Although initially developed for detection of small amounts of cDNA, we have found the method to be highly effective when preceding routine Northern blot analysis, enabling rapid discrimination of connexins belonging to group I from those belonging to group II in both large and small amounts of samples. In addition, the present methodology could be adapted to other gene families with high sequence conservation.

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