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# Molecular Cloning and Expression of Rat Connexin40, a Gap Junction Protein Expressed in Vascular Smooth Muscle

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**Summary.** Gap junctions contain intercellular channels which are formed by members of a group of related proteins called connexins. Connexins contain conserved transmembrane and extracellular domains, but unique cytoplasmic regions which may provide connexin-specific physiologic properties. We used polymerase chain reaction (PCR) amplification and cDNA library screening to clone DNA encoding a novel member of this gene family, rat connexin40 (Cx40). The derived rat Cx40 polypeptide contains 356 amino acids, with a predicted molecular mass of 40,233 Da. Sequence comparisons suggest that Cx40 is the mammalian homologue of chick connexin42, but it has predicted cytoplasmic regions that differ from previously described mammalian connexins. Southern blots of rat genomic DNA suggest that Cx40 is encoded by a single copy gene containing no introns within its coding region. Northern blots demonstrate that Cx40 is expressed in multiple tissues (including lung, heart, uterus, ovary, and blood vessels) and in primary cultures and established lines of vascular smooth muscle cells. Cx40 is coexpressed with connexin43 in several cell types, including A7r5 cells, which contain two physiologically distinct gap junctional channels. To demonstrate that Cx40 could form functional channels, we stably transfected communication-deficient Neuro2A cells with Cx40 DNA. These Cx40-transfected cells showed intercellular passage of microinjected Lucifer yellow CH. The expression of multiple connexins (such as Cx40 and Cx43) by a single cell may provide a mechanism by which cells regulate intercellular coupling through the formation of multiple channels.

**Key Words:** gap junction · intercellular communication · connexin40 · vascular smooth muscle · A7r5 cell line

## Introduction

Gap junctions contain the low resistance channels which coordinate tissue function by allowing cell-to-cell passage of ions and small molecules. In electrically excitable tissues such as myocardium and smooth muscle, electrical coupling through gap junctions facilitates action potential propagation. The blood vessel wall is a tissue where gap junctions may perform a number of metabolic and electrical functions (Larson, 1988). Experiments have suggested that vascular muscle gap junctions may coordinate vasomotor responses to humoral agents by mediating cell-to-cell spread of a dilating or constricting wave (Segal & Duling, 1986; Segal, Daman & Duling, 1989).

Recent biochemical and molecular studies have demonstrated that gap junctional channels are formed by members of a family of proteins called connexins (reviewed by Beyer, Paul & Goodenough, 1990). Topological studies have suggested that the extracellular and transmembrane regions of all connexins are highly conserved, while the cytoplasmic domains are nearly unique to each protein. These connexin-specific sequences may endow channels with unique physiologic properties. Indeed, in expression studies, cloned connexin DNAs have produced channels with differing biophysical characteristics, including unitary conductance and voltage dependence (Ebihara et al., 1989; Swenson et al., 1989; Werner et al., 1989; Eghbali, Kessler & Spray, 1990; Fishman, Spray & Leinwand, 1990).

We have been trying to characterize molecularly the gap junctions formed between cells of the vessel wall. Larson, Haudenschild and Beyer (1990) reported that endothelial cells, vascular smooth muscle cells, and pericytes all express the same gap junction protein, connexin43 (Cx43). Lash, Critser and Pressler (1990) cloned Cx43 cDNA from bovine aortic smooth muscle cells. Yet, Moore, Beyer and Burt (1991) recently demonstrated that A7r5 rat aortic smooth muscle cells express gap junctional channels which have two different unitary conductances (~36 and ~90 pS) and which have some different responses to second messengers than other Cx43-expressing cells (Burt & Spray, 1988).

We hypothesized that the complexity of the gap junctional channels observed in these cells might be due to the expression of an additional, previously

unidentified connexin protein. We now report the identification of a novel connexin sequence, rat connexin40 (Cx40), which is expressed in a number of different tissues and by A7r5 cells and primary cultures of vascular smooth muscle. We also provide a functional demonstration that the protein encoded by this sequence is capable of forming cell-to-cell channels.

## Materials and Methods

### DNA Cloning and Sequencing

The initial fragment of canine Cx40 was amplified from dog heart RNA using the reverse transcriptase modification of the polymerase chain reaction (PCR) with the exact buffers and temperatures described by Kawasaki (1990). Total RNA was isolated from dog ventricle according to Chomczynski and Sacchi (1987). First strand cDNA was synthesized in a 20-$\mu$l reaction using 1 $\mu$g of RNA and random hexanucleotide primers; the reaction was diluted to 100 $\mu$l and amplified through 35 cycles with a degenerate/consensus primer corresponding to a conserved sequence in the first connexin extracellular loop (AACACTCTGCAGCCTG-GCTGT$^{A}/_{G}$A$^{G}$/C/$_{A}$AACGTCTGCTA$^{C}/_{T}$GAC) and an antisense primer corresponding to the second connexin extracellular loop (AGCATGATGATCATGAAGA$^{T}/_{C}$GGT$^{T}/_{C}$TCNGTGGG). Restriction sites (Xho I and Bcl I, respectively) were incorporated into the primers to facilitate subcloning into Bluescript vectors (Stratagene, San Diego, CA). The DNA fragment of canine Cx40 was used to screen a rat aorta cDNA library (Clontech, Palo Alto, CA) by hybridization according to Beyer et al. (1987) to isolate rat sequence.

The full coding sequence of rat Cx40 was amplified from rat genomic DNA by PCR (Saiki et al., 1988) using a sense amino terminal primer (TGCTGCAAGCTTAAGATGGG$^{A}/_{T}$GA$^{C}$/$_{T}$TGGAG$^{C}/_{T}$TTCCTGC, which contains a Hind III site and is degenerate to match dog Cx40 and chick Cx42) and an antisense carboxyl terminal primer (CTAAGATCTAGATCACACTG A$^{C}$/$_{T}$AG$^{A}/_{G}$TCATCTGACCT, which contains a Bgl II site and is degenerate to match dog and rat Cx40).

DNA sequencing was performed using plasmid templates, Sequenase enzymes (USB, Cleveland, OH), and oligonucleotide primers as previously described (Beyer, 1990). DNA sequence acquisition and initial analysis was performed using Microgenie (Beckman Instruments, Palo Alto, CA) software running on an IBM-compatible microcomputer (Queen & Korn, 1984). Protein sequence alignments and comparisons were performed using the CLUSTAL program (Higgins & Sharp, 1988) with the PC Gene software (Intelligenetics, Mountain View, CA)

### Southern and Northern Blots

Adult rat DNA was isolated, digested with restriction enzymes, electrophoresed in 1% agarose gels and transferred to nylon membranes as previously described (Lang et al., 1991).

Total cellular RNA was prepared from cells or tissues according to Chomczynski and Sacchi (1987), separated on formaldehyde/agarose gels, and transferred to nylon membranes as previously described (Beyer et al., 1987). Hybridization was performed using specific $^{32}$P-labeled DNA probes prepared using random hexanucleotide primers and the Klenow fragment of DNA polymerase I (Beyer, 1990).

### Cell Cultures

Rat aorta smooth muscle A7r5 and A10 cells, mouse $BC_3H1$ myoblasts, rat $C_6$ glioma cells, SK Hep1 human hepatoma cells, and mouse Neuro2A (N2A) neuroblastoma cells were obtained from the American Type Culture Collection (Rockville, MD). Normal rat kidney fibroblasts (NRK) were the gift of Dr. Stuart Kornfeld (Washington University, St. Louis, MO). Rat Morris hepatoma cells ($MH_1C_1$) were the gift of Dr. Alan L. Schwartz (Washington University, St. Louis, MO). N2A and SK Hep1 cells were grown in Minimal Essential Medium (GIBCO Laboratories, Grand Island, NY) supplemented with 10% heat-inactivated (56°C for 30 min) fetal calf serum (JRH Biosciences, Lenexa, KS), 1X nonessential amino acids (GIBCO Laboratories), 2 mM L-glutamine, 100 U/ml penicillin and 100 $\mu$g/ml streptomycin (GIBCO Laboratories). All other cells were grown in Dulbecco's Modified Eagle Medium (GIBCO Laboratories) supplemented with 10% fetal calf serum (JRH Biosciences, Lenexa, KS), 100 U/ml penicillin and 100 $\mu$g/ml streptomycin (GIBCO Laboratories). Primary cultures of bovine and rat aortic smooth muscle cells and of bovine aortic and human umbilical vein endothelial cells were grown as described previously (Larson et al., 1990).

### Functional Expression Studies

The coding sequence of dog connexin40 was cloned into the multiple cloning site of pRc/RSV-neo (Invitrogen, San Diego, CA). N2A cells in 60-mm dishes were transfected with 20 $\mu$g linearized plasmid using the Lipofectin reagent (GIBCO/BRL, Gaithersburg, MD) according to the manufacturer's directions, and stable, neomycin-resistant colonies were selected in 0.5 mg/ml G418 (GIBCO/BRL, Gaithersburg, MD). Connexin expression was verified by Northern blotting of total RNA prepared from selected clones.

Functional intercellular coupling was verified by dye transfer studies performed essentially according to Larson, Carson and Haudenschild (1987). A 10% solution of Lucifer yellow CH (Sigma, St. Louis, MO) was directly microinjected into cells using a Nikon pico-injector and visualized with a Nikon Diaphot inverted microscope equipped with epifluorescence and Hoffman modulation contrast optics.

## Results

### Cloning of Rat Connexin40

All connexins studied to date have single copy genes which lack introns in their coding sequences (Miller, Dahl & Werner, 1988; Fishman et al., 1991) and which contain highly conserved sequences corresponding to four transmembrane and two extracellular domains within the proteins (Beyer, Paul & Goodenough, 1990). We took advantage of these characteristics and used reverse transcriptase/poly-

merase chain reaction (RT/PCR) to attempt to isolate novel connexin sequences expressed in the cardiovascular system. Using oligonucleotide primers corresponding to the two conserved extracellular regions, we amplified several 350–500 bp sequences from canine ventricular RNA which were subcloned and sequenced. One 417-bp sequence clearly corresponded to a portion of a previously unidentified connexin. High stringency Northern blots performed using this sequence as probe identified a mRNA of ~2.5 kb in dog ventricle, dog aorta, and dog coronary artery and a very abundant mRNA of ~3.3 kb in A7r5 cells (see Fig. 4, lanes *K-N*). In the course of other studies, we found that the canine sequence corresponded to bases 205–622 of dog connexin40 (Cx40) (Kanter, Saffitz & Beyer, 1992).

We sought to identify rat cDNA clones corresponding to this novel connexin sequence. We identified a 835-bp clone in a rat aorta cDNA library by hybridization screening, which corresponds to bases 547–1382 in Fig. 1. However, rescreening of this library or of other rat cDNA libraries did not identify longer clones. Therefore, we used the PCR with an amino terminal primer matching dog Cx40 and chick Cx42 and a carboxyl terminal primer from the 835-bp rat sequence to amplify the homologous sequence (bases 1–1071, Fig. 1) from rat genomic DNA. Its sequence overlapped and matched our partial cDNA perfectly.

The rat DNA sequence encodes a polypeptide of 356 amino acids with a predicted molecular mass of 40,233 Da, rat Cx40. Comparison of rat Cx40 to the amino acid sequences of other connexins (Fig. 2) demonstrates that this protein is clearly related to other connexins. It is most similar to dog Cx40 (83% identical) and chick connexin42 (71% identical), suggesting that these are the homologous connexins in these other species. Rat Cx40 contains 45% identical amino acids to rat Cx43; most of the differences between rat Cx40 and Cx43 occur in the predicted cytoplasmic regions. The published sequences of other connexins are somewhat less homologous, but also contain many identical residues in predicted transmembrane and extracellular regions.

## Southern Blots

Southern blots of restriction enzyme digested rat genomic DNA were hybridized with rat Cx40 probes. As shown in Fig. 3, the 835-bp rat cDNA probe hybridized only to single bands in most of the enzyme digests, suggesting that rat Cx40 is a single copy gene. Two bands were observed only for Hinc II, which were predicted by an internal restriction site within the probe. The full coding sequence produced by PCR from genomic DNA predicts a 739-bp Pst I fragment which is identified on the blot. Further blots with a more 5′ probe gave similar results (*data not shown*). These blots in addition to the PCR amplification suggest that there are no introns present within this sequence. The uniqueness of the Cx40 gene is confirmed by the difference between this blot and published Southern blots probed with other rat connexins (Miller et al., 1988; Zhang & Nicholson, 1989; Lang et al., 1991).

## Expression of Connexin40 mRNA

To determine the pattern of expression of Cx40, we performed Northern blot analysis of total RNA isolated from many organs and tissues isolated from mouse, rat, dog, or rabbit. Figure 4 shows filters probed at high stringency with the probes for rat Cx40 (lanes *A–J*) and dog Cx40 (lanes *K–N*). This figure demonstrates that Cx40 mRNA was most abundantly expressed by the A7r5 cells, but also was expressed in substantial amounts in heart, uterus, ovary, aorta, and coronary artery. Cx40 transcript was also very abundant in lung RNA and was detectable in kidney, stomach, and rabbit gastric smooth muscle (*not shown*). No Cx40 hybridization was detected in RNA from brain, spleen, or placenta (*not shown*). The Cx40 transcript in rat was considerably larger than that in dog (3.3 *vs.* 2.5 kb), which presumably reflects differences in untranslated portions of the mRNAs.

Since Cx40 mRNA was expressed in many different tissues and since its general distribution appeared similar to that previously demonstrated for Cx43 (Beyer et al., 1987), we examined the expression of Cx40 and Cx43 by established cell lines and primary cultures of vascular cells. Identical Northern blots of total RNA prepared from the different cells were hybridized with specific DNA probes for rat Cx40 or rat Cx43 (Fig. 5). Cx43 mRNA was detected in RNA from the primary cultures of endothelial and vascular smooth muscle cells, as well as in cell lines of many different sources, including vascular smooth muscle (A7r5, A10), skeletal myoblasts ($BC_3H1$), fibroblasts (NRK), and glioma cells ($C_6$). Cx43 hybridization was not detected in RNA derived from N2A cells or from SK Hep1 or $MH_1C_1$ cells (*data not shown*). In contrast, Cx40 hybridization was most abundant in the A7r5 cells and was less abundant in the related A10 cells. Cx40 mRNA was detected in primary cultures of bovine aortic smooth muscle cells, although expression appeared to fall with successive passages. Cx40 was not detected in any of the other cells tested, except for the SK Hep1 cells (*not shown*) which gave only a weak band. A Cx40 hybridizing band was also

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    ATGGGTGACTGGAGCTTCCTGGGGGAGTTCCTGGAGGAGGTCCACAAGCACTCTACGGTC   60
  1 M  G  D  W  S  F  L  G  E  F  L  E  E  V  H  K  H  S  T  V

    ATCGGCAAGGTCTGGCTCACCGTCCTGTTCATTTTCCGCATGCTGGTCCTCGGCACCGCT  120
 21 I  G  K  V  W  L  T  V  L  F  I  F  R  M  L  V  L  G  T  A

    GCTGAGTCCTCCTGGGGAGATGAGCAGGCTGATTTCCGGTGTGATACCATTCAGCCTGGT  180
 41 A  E  S  S  W  G  D  E  Q  A  D  F  R  C  D  T  I  Q  P  G

    TGCCAAAATGTCTGCTACGACCAAGCCTTCCCCATCTCCCACATTCGTTACTGGGTACTG  240
 61 C  Q  N  V  C  Y  D  Q  A  F  P  I  S  H  I  R  Y  W  V  L

    CAGATCATCTTTGTGTCCACACCATCTCTGGTGTACATGGGCCACGCCATGCACACTGTG  300
 81 Q  I  I  F  V  S  T  P  S  L  V  Y  M  G  H  A  M  H  T  V

    CGCATGCAGGAAAAGCAGAAGCTGCGGGAGGCTGAGAAAGCTAAAGAGGCCGGTGGCACC  360
101 R  M  Q  E  K  Q  K  L  R  E  A  E  K  A  K  E  A  G  G  T

    GGCACCTATGAGTACTTGGCGGAGAAAGCCGAGCTGTCCTGCTGGAAAGAGGTGAACGGG  420
121 G  T  Y  E  Y  L  A  E  K  A  E  L  S  C  W  K  E  V  N  G

    AAGATTGTCCTCCAGGGCACCCTACTCAACACCTATGTCTGCACCATTCTCATCCGCACC  480
141 K  I  V  L  Q  G  T  L  L  N  T  Y  V  C  T  I  L  I  R  T

    GCTATGGAGGTGGCCTTCATGGTGGGCCAGTACCTCCTCTATGGGATCTTCCTGGACACC  540
161 A  M  E  V  A  F  M  V  G  Q  Y  L  L  Y  G  I  F  L  D  T

    CTGCATGTCTGCCGCAGAAGTCCCTGTCCCCACCCCGTCAACTGTTATGTCTCGAGGCCC  600
181 L  H  V  C  R  R  S  P  C  P  H  P  V  N  C  Y  V  S  R  P

    ACAGAGAAGAACGTCTTCATTGTCTTTATGATGGCTGTGGCTGGACTGTCTCTGTTTCTC  660
201 T  E  K  N  V  F  I  V  F  M  M  A  V  A  G  L  S  L  F  L

    AGCCTGGCTGAACTCTACCACCTGGGCTGGAAGAAGATCCGACAGCGCCTGGCCAAGTCA  720
221 S  L  A  E  L  Y  H  L  G  W  K  K  I  R  Q  R  L  A  K  S

    CGGCAGGGTGACAAGCACCAGCTTCTTGGCCCCTCCACCAGCCTGGTCCAGGGCCTCACT  780
241 R  Q  G  D  K  H  Q  L  L  G  P  S  T  S  L  V  Q  G  L  T

    CCTCCTCCTGACTTCAACCAGTGCCTAAAGAACAGCCCAGATGAGAAATTCTTCAGTGAC  840
261 P  P  P  D  F  N  Q  C  L  K  N  S  P  D  E  K  F  F  S  D

    TTCAGTAATAACATGGGCTCCCGGAAGAATCCAGACCCTCTGGCCACTGAGGAAGTGCCA  900
281 F  S  N  N  M  G  S  R  K  N  P  D  P  L  A  T  E  E  V  P

    AACCAGGAGCAGATCCCGGAGGAAGGTTTCATCCACACACAGTATGGCCAGAAGCCCGAG  960
301 N  Q  E  Q  I  P  E  E  G  F  I  H  T  Q  Y  G  Q  K  P  E

    CAGCCCAGTGGCGCCTCTGCAGGCCACCGCTTTCCTCAGGGCTACCACAGTGACAAGCGC 1020
321 Q  P  S  G  A  S  A  G  H  R  F  P  Q  G  Y  H  S  D  K  R

    CGCCTTAGTAAGGCCAGCAGCAAAGCAAGGTCAGATGACCTGTCAGTGTGACCCTCCTCT 1080
341 R  L  S  K  A  S  S  K  A  R  S  D  D  L  S  V  .

    TAGGGAGGACCAGTCCCAGAAGGAAGCCAAGGAGGGTGAGGCAGGACAGAAGCGGTCTCT 1140
    CTGACTGTATACCTTCCTCAGCACCTTCTCTCCCGGGTCTGGATGGTGTTGCTCATTAAT 1200
    CCTCCAAATTTCAGTTCTGAGCCGTATTCTCAAAGATTGGGCTACTCCTCAAATCCCCAA 1260
    CCCCACATCCTCTACCAGTATTCGCAGTGAAGACTTTCAAGTGATTTAGTGATATGGTGG 1320
    GAGCAGGAATGGGAACCTTGGCAAAATCTGGTCTGGGTAGGACAGACATGAAGATTCGAC 1380
    CG                                                            1382
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**Fig. 1.** Sequence of rat connexin40. The composite nucleotide sequence derived from rat aortic cDNA and by PCR from genomic DNA is shown in light type with residues numbered on the right. The derived amino acid sequence is shown in bold face type.

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Rat Cx40    MGDWSFLGEFLEEVHKHSTVIGKVWLTVLFIFRMLVLGTAAESSWGDEQA   50
Dog Cx40    **************************************************   50
Chk Cx42    ********************V**********************GPL*****S   50
Rat Cx43    *****A**KL*DK*QAY**AG*****S******I*L****V**A*****S   50

Rat Cx40    DFRCDTIQPGCQNVCYDQAFPISHIRYWVLQIIFVSTPSLVYMGHAMHTV  100
Dog Cx40    **Q***M****G************************************  100
Chk Cx42    **M***Q****E*****K******V*F**********************  100
Rat Cx43    A***N*Q****E*****KS*****V*F*********V*T*L*LA*VFYVM  100

Rat Cx40    RMQEKQKLREAE-KAKEAGGTGTYEY------LAEKAELSCWKEVNGKIV  143
Dog Cx40    *****RNV*K**-R*****A-*S***-----PV********E****R**  143
Chk Cx42    **E**R*MK***-REAQEMKNSGDT*YQQKCPV***T*****D*SG***I  149
Rat Cx43    *KE**LNKK*E*L*VAQTD*VNVEMHLKQ----I*IKKFKYGI*EH**VK  146

Rat Cx40    LQGTLLNTYVCTILIRTAMEVAFMVGQYLLYGIFLDTLHVCRRSPCPHPV  193
Dog Cx40    **********S*****T*****I************************** 193
Chk Cx42    *R*S******YS********I**I****I******E**YI*Q*A****** 199
Rat Cx43    MR*G**R**IIS**FKSVF****LLI*WYI**FS*SAVYT*K*D****Q*  196

Rat Cx40    NCYVSRPTEKNVFIVFMMAVAGLSLFLSLAELYHLGWKKIRQRL---A--  238
Dog Cx40    ****************L***A****************L***F---V--  238
Chk Cx42    **************I******V****************AKE*C---SRA  246
Rat Cx43    D*FL******TI**I**LV*SLV**A*NII**FYVFF*GVKD*VKGRSDP  246

Rat Cx40    -KSRQGDKH-QL-LGPS--------TSLVQGLTP--PPDFNQCLKNSPDE  275
Dog Cx40    -**G**MAEC**-P***--------AGI**NC**--**********G*GG  276
Chk Cx42    Y*PSPSTAPRR*ESA*Q--------VERA*MY**--********-A**NG  285
Rat Cx43    YHATT*PLSPSKDCGSPKYAYFNGCS*PTAP*S*MS**GYKL-VTGDRNN  295

Rat Cx40    KFFSDFSNNMGSRKNPDPLATEEVPNQEQIPEEG-FIHT-QYGQKPEQPS  322
Dog Cx40    ***NP***K*A*QQ*T*N****Q*QG**P**G**-**NI-R*A****V*N  324
Chk Cx42    **I*P***KMA*QQ*TANF***R*HS**DAAG**P*MKS-S*MES**VA*  334
Rat Cx43    SSCRNYNKQASEQNWANYS*EQNRMG*AGSTISNSHAQPFDFPDDNQNAK  345

Rat Cx40    GASAGHRFPQGYHSDKRRLSKAS----SKARSDDLSV   355
Dog Cx40    G**P***L*H**Q**********----**********   357
Chk Cx42    E-C*APAL*ES*FNE***F****-RAS**********   369
Rat Cx43    KVA***ELQPLAIV*Q*PS*R**SRAS*RP*P***EI   382
```

**Fig. 2.** Comparison of rat Cx40 to several closely related connexins. The derived amino acid sequences of rat Cx40 and those of dog Cx40 (Kanter et al., 1992), chick Cx42 (Beyer, 1990), and rat Cx43 (Beyer et al., 1987) are shown as optimally aligned by CLUSTAL (Higgins & Sharp, 1988). Residues that are identical to their counterparts in rat Cx40 are represented by asterisks; nonidentical residues are listed in single letter code. Dashes represent spaces added to optimize alignment.

detected in poly-adenylated RNA from A7r5 cells selected by oligo-dT cellulose chromatography (*data not shown*).

### Functional Expression of Connexin40

To verify that Cx40 was indeed capable of forming functional cell-to-cell channels, we stably transfected the communication-deficient cell line N2A with a construct containing the coding sequence of dog Cx40 under the control of a Rous sarcoma virus promoter. The N2A cells make no known connexin mRNAs or proteins, contain no gap junctional channels detectable by the double whole-cell patch-clamp technique, and have been successfully used for the expression of several connexin sequences (Veenstra et al., 1992; R.D. Veenstra, H.-Z. Wang, E. M. Westphale, and E.C. Beyer, *in preparation*). Northern blot analysis of RNA prepared from the transfected cells demonstrated that they expressed Cx40 mRNA, while no hybridization was detected in cells transfected with vector alone (Fig. 6). The Cx40 mRNA in the transfected cells was smaller than the authentic mRNA in A7r5 cells, because it contained only coding sequence and a small amount of vector sequence.

The Cx40-transfected cells were tested for functional intercellular coupling by a dye transfer assay.

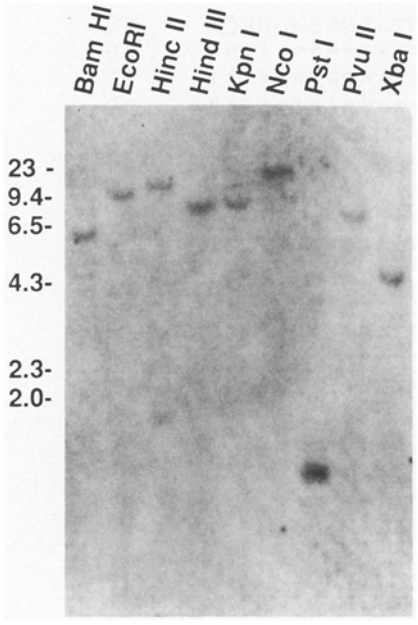


**Fig. 3.** Southern blot analysis of rat genomic DNA. DNA was digested with several restriction endonucleases as indicated, separated by agarose electrophoresis, and transferred to nylon membranes. The blot was hybridized with a cDNA probe corresponding to nucleotides 547–1382 of the rat Cx40 sequence. Lambda phage DNA digested with Hind III was used as molecular mass standards with size in kb indicated to the left of the blot. For all enzymes, hybridization produced only one or two bands. Multiple bands were observed only where the sequence predicted internal restriction sites (Hinc II).

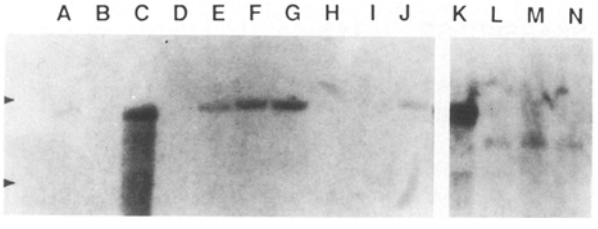


**Fig. 4.** Northern blots demonstrating tissue distribution of expression of Cx40 mRNA. Total cellular RNA was prepared from cells or tissues, separated on formaldehyde/agarose gels (10 $\mu$g/lane) and prepared for Northern blotting as described in Materials and Methods. Blots were then incubated with specific $^{32}$P-labeled DNA probes corresponding to nucleotides 547–1382 of rat Cx40 (lanes *A–J*) or nucleotides 205–622 of dog Cx40 (Kanter et al., 1992) (lanes *K–N*). RNAs were prepared from mouse kidney (*A*), mouse brain (*B*), A7r5 cells (*C*,*K*), mouse liver (*D*), mouse heart (*E*), rat uterus (*F*), rat ovary (*G*), mouse spleen (*H*), mouse stomach (*I*), rat heart (*J*), dog ventricle (*L*), dog aorta (*M*), and dog coronary (*N*). Arrowheads indicate the migration of 18S and 28S rRNAs.

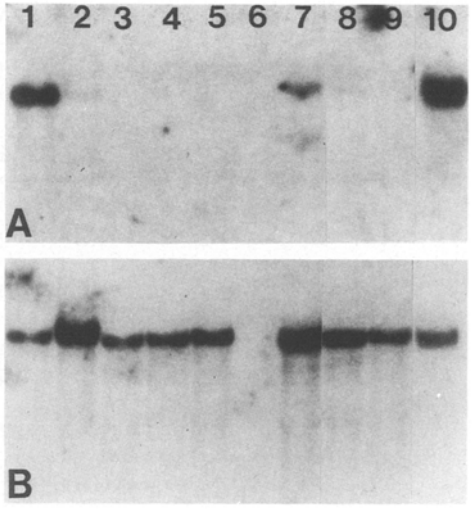


**Fig. 5.** Northern blots comparing expression of Cx40 and Cx43 by cultured cells. Total RNA was prepared from A7r5 (*1*, *10*), A10 (*2*), $BC_3H1$ (*3*), NRK (*4*), $C_6$ glioma (*5*), N2A (*6*), first passage bovine aortic smooth muscle (*7*), third passage bovine aortic smooth muscle (*8*), and bovine aortic endothelial (*9*) cells. Two identical blots were prepared and hybridized with specific cDNA probes for rat Cx40 (*A*) or rat Cx43 (*B*).

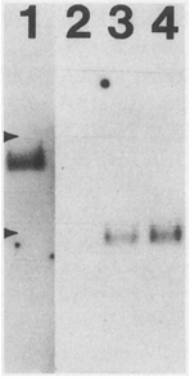


**Fig. 6.** Northern blots demonstrating expression of Cx40 in transfected cells. Total RNA was prepared from A7r5 cells (*1*), N2A cells transfected with pRc/RSV-neo vector alone (*2*), or from two different clones of N2A cells transfected with pRc/RSV-neo constructs containing the dog Cx40 coding sequence. Arrowheads indicate the migration of 18S and 28S rRNAs.

In over 40 injections of untransfected N2A cells or N2A cells transfected with vector alone, we have never seen significant transfer of dye. In most cases only the single injected cell was seen to be loaded with dye. In a small number of cases (<10%), two cells were loaded with dye, which we interpret as the simultaneous injection of both cells. In contrast, we have performed over 50 injections of two different Cx40-expressing N2A clones (transfectants), and we have observed transfer of dye to at least one neighbor cell in about 50% of cases. In five cases, microinjected Lucifer yellow CH readily spread from the injected cell into multiple surrounding cells, as illustrated in Fig. 7.

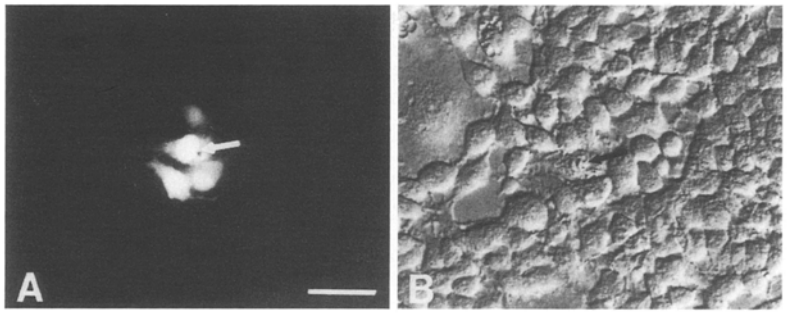


**Fig. 7.** Functional demonstration of intercellular communication by N2A cells stably transfected with Cx40 DNA. Lucifer yellow CH microinjected into a single cell (arrows) in a confluent dish of N2A cells stably transfected with Cx40 spreads to several adjacent cells. Cells were visualized by fluorescence (*A*) and Hoffman modulation contrast (*B*) microscopy. Bar, 40 μm.

## Discussion

In the present study, we have identified and sequenced DNA corresponding to a novel gap junction protein, rat Cx40. Cx40 mRNA expression was observed in a number of locations, most notably in vascular smooth muscle lines and in primary cultures. The expression of Cx40 appeared to diminish with successive passages of the primary cells in culture, which could have been due to the phenomenon of "phenotypic modulation" of these cells. It has been well documented that once in culture, vascular smooth muscle cells usually change from a "contractile" to a "synthetic/proliferative" phenotype with the loss of many differentiated characteristics, including some cell surface receptors and smooth muscle-specific contractile protein isoforms (Chamley-Campbell, Campbell & Ross, 1979; Larson et al., 1984). The clonal cell line A7r5 may have had Cx40 expression fixed during adaptation to culture.

The present study provides convincing evidence that the A7r5 cell line, which contains two populations of gap junctional channels (36 and 90 pS) (Moore et al., 1991), expresses two different connexins, Cx40 and Cx43. We may soon be able to dissect the properties conferred by each of these channel proteins, by specifically blocking the production of each isoform with antisense oligonucleotides or by individually expressing each connexin in the communication-deficient N2A cells. It is reasonable to speculate that the 36-pS channel in the A7r5 cells might be formed of Cx40, since a few 30-pS channels have been detected in SK Hep1 cells (Eghbali et al., 1990) where we find a low level of Cx40 mRNA.

The co-expression of Cx40 and Cx43 by vascular smooth muscle cells may represent a general strategy which cells use to regulate intercellular communication: the expression of multiple gap junction proteins which form channels with different physiologic properties. It has been well documented that hepatocytes express both Cx32 and Cx26 (Traub et al., 1989; Zhang & Nicholson, 1989). Chick embryo heart cells contain several different channels whose prominence varies with development (Veenstra, 1991) and express Cx42, Cx43, and Cx45 (Beyer, 1990). Mammalian ventricular myocytes express Cx40, Cx43, and Cx45 (Kanter et al., 1992).

Different connexins expressed within a cell may have different responses to intracellular signaling mechanisms, especially those involving protein phosphorylation. Cx43 has been demonstrated to be a phosphoprotein, with phosphorylation of serine and tyrosine residues (Crow et al., 1990; Filson et al., 1990; Musil, Beyer & Goodenough, 1990*a*). Musil et al. (1990*b*) suggested that mature serine phosphorylation of Cx43 correlated with communication competence. On the other hand, tyrosine phosphorylation of Cx43 was accompanied by a loss of intercellular coupling (Crow et al., 1990; Filson et al., 1990). The carboxyl terminus of Cx40 is very rich in serines with a number of potential cyclic nucleotide-dependent kinase or protein kinase C phosphorylation sites; this region is similar, but not identical to the corresponding region in Cx43. In Cx43, tyrosine 265 has been implicated as the site of phosphorylation by $pp60^{v\text{-}src}$ which results in loss of coupling (Swenson et al., 1990); Cx40 does not contain a corresponding site.

Thus, an exciting direction for investigations of intercellular communication will be the examination of mechanisms by which cells regulate the expression of multiple connexins and the post-translational modification of those channel proteins.

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The nucleotide sequence for rat Cx40 has been submitted to the Genbank and EMBL databases with the accession number M83092.

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