Mouse Connexin37: Cloning and Functional Expression of a Gap Junction Gene Highly Expressed in Lung

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Abstract. The coding sequence (333 amino acids) of a new connexin protein, designated mouse connexin37 (Cx37 or Cx37.6) due to the deduced theoretical molecular mass of 37.600 kD, has been determined from cDNA and genomic clones. As seen in other connexins, its gene has no introns within the coding region and the deduced amino acid sequence is predicted to have similar topology to other connexins that form intercellular channels. The amino acid sequence of mouse Cx37 is most similar to rat connexin43 (59% identity) and *Xenopus* connexin38 (66% identity) when compared from the NH_2 terminus to the end of the fourth putative transmembrane region. When expressed in *Xenopus* oocytes Cx37 forms functional intercellular channels that exhibit more sensitive and rapid gating in response to voltage than any previously characterized vertebrate gap junction. Under stringent conditions the Cx37 cDNA hybridizes to an mRNA of 1.7 kb that is found highly abundant in lung and to progressively lesser extents in brain, kidney, skin, spleen, liver, intestine, and heart. Embryonic brain, kidney, and skin express two to fivefold higher levels of the Cx37 transcript than the corresponding adult tissues. Cx37 transcripts were also found to increase two to threefold in response to retinoic acid treatment of cultured embryonic carcinoma F9 cells.

CONNEXINS are the protein subunits of gap junctional cell to cell channels. So far four different connexin cDNAs have been cloned from rat tissues: connexin32 (Cx32) (Paul, 1986; Kumar and Gilula, 1986; Heynkes et al., 1986), connexin26 (Cx26) (Zhang and Nicholson, 1989) from liver, connexin43 (Cx43) (Beyer et al., 1987) from heart, and connexin46 (Cx46) (Beyer et al., 1988) from lens. Another member of the connexin family (M_r 70,000) has been identified biochemically in sheep eye lens (Kistler et al., 1988) and several other connexins have been cloned in chicken (Cx42, Cx43, and Cx45; Beyer, 1990) and frog (Cx30, Cx38, and Cx43; Ebihara et al., 1989; Gimlich et al., 1988, 1990). The nomenclature originally proposed by Beyer et al. (1987, 1990) and used throughout this manuscript in abbreviated form, uses calculated molecular mass in kilodaltons to differentiate family members and thus makes no overt assumptions as to the relatedness within the family (see Gimlich et al., 1990; Risek et al., 1990). The amino acid sequences of all known connexins show a similar topology suggested by their hydropathy plots. There are four putative transmembrane regions of which the third one contains polar residues suggestive of an amphipathic helix. This has led to the proposal that six of these helixes (one per subunit) may form the gap junction channel (for review see Beyer et al., 1990; Willecke and Traub, 1990). Results with different antipeptide antibodies and assays of membrane proteolysis protection support the topological model and suggest that both the NH_2- and COOH-terminal sequence of each connexin subunit face the cytoplasm (Milks et al., 1988; Hertzberg et al., 1988; Beyer et al., 1989; Yancey et al., 1989). In comparisons of the known connexin sequences, it is notable that these cytoplasmic domains are most variable while those positions of the sequence involved in channel structure (in membrane spans and extracellular loops) are most strictly conserved.

The genes for rat connexin32 and human connexin43 have been described (Miller et al., 1988; Fishman et al., 1990). Both genes contain an intron in the untranslated region upstream of the notably intron-less coding region. Connexin genes have recently been shown not to be largely clustered, with six different genes and one pseudogene being mapped to five human chromosomes (Willecke et al., 1990*b*).

Although connexins are expressed in a cell type–specific manner there appears to be considerable overlap in their distributions (Beyer et al., 1989; Dermietzel et al., 1989; Zhang and Nicholson, 1989). Indeed, in hepatocytes both Cx32 and Cx26 are found in the same gap junctional plaque (Nicholson et al., 1987). Since intracellular communication between hepatocytes can be strongly inhibited by microinjected antibodies to Cx32 and Cx26 it has been suggested that heteromeric gap junction channels may exist in these cells (Traub et al., 1989). Interactions between different connexins have been demonstrated directly in *Xenopus* oocytes where each cell of the pair expresses a distinct connexin. Heterotypic in-

teractions between rat Cx43 and Cx32, or *Xenopus* Cx38 (Swenson et al., 1989; Werner et al., 1989) as well as the more physiologically relevant case of rat Cx32 and Cx26 (Barrio et al., submitted) have been documented in this manner. However, it is clear that individual connexins can also form intercellular channels alone when expressed in *Xenopus* oocytes (Dahl et al., 1987; Swenson et al., 1989) or in coupling deficient human hepatoma cells (Eghbali et al., 1990; Fishman et al., 1990).

Here we describe isolation and characterization of the coding sequence for mouse Cx37, whose transcripts are particularly abundant in lung. Like other members of the connexin family, Cx37 shows relatively high homology with other connexins in those sections, coding for the two presumably extracellular domains and three of the four putative transmembrane regions. Little or no homology to other connexins is found in the presumably cytoplasmic loop connecting the second and third hydrophobic regions nor in the long region coding for the presumably intracellular COOH-terminal half of the polypeptide chain.

Materials and Methods

DNA Libraries

The cDNA library (8×10^6 recombinants, average size of insert DNA 1.7 kb), in lambda gt11, from adult mouse brain minus cerebellum was a gift of Dr. D. Bartels and Dr. W. Wille (Institut für Genetik, Universität Köln, Germany). The mouse genomic library was prepared by Ms. Rita Lange and Dr. W. Wille after partial digestion of mouse sperm DNA with SauIIIa, size fractionation (10–20 kb) of the DNA and ligation into the substitution vector EMBL3 cut with BamHI. The genomic library has a complexity of $\sim 3 \times 10^6$ independent phages (average size of insert DNA 17–18 kb).

Isolation of Mouse Cx37 cDNA

The mouse brain cDNA library (500,000 lambda gt11-plaques) was screened on 10 filters (132 mm, Hybond-N; Amersham International, Amersham, UK) with a rat liver Cx32 cDNA clone (BamHI–HindIII fragment) (Heynkes et al., 1986) that had been labeled by random priming (Amersham International). Prehybridization of the filters was carried out overnight at 38°C in 5×SSC, 5× Denhardt's solution, 0.5% SDS, and 50% formamide (1×SSC is 0.15 M NaCl containing 0.015 M sodium citrate; Denhardt's solution: 0.02% (wt/vol) BSA, 0.2% (wt/vol) Ficoll, and 0.02% (wt/vol) polyvinylpyrollidone). Then the labeled DNA probe (50 ml with 6×10^6 cpm; specific activity: 2.4×10^8 cpm/μg) was added and allowed to hybridize for 2 d at 38°C. Filters were subsequently washed for 1 h at 50°C in 2×SSC, 1% SDS and twice for 1 h at 50°C in 1×SSC, 0.1% SDS prior to exposure to x-ray film for 10 d at −80°C. Plaques hybridizing to the probe were replaced and replicas were obtained on filters that had been prehybridized in a solution of 0.25% powdered milk, 6×SSC, and 50% formamide. The washing steps were the same as described for identification of the plaques. DNA from three plaques was purified after lysis of *Escherichia coli* Y1090 host bacteria by phenol extraction and adsorption to glass powder (Vogelstein and Gillespie, 1979). After EcoRI digestion the isolated DNA was subcloned into pUC19 and sequenced on both strands by a modification (Tabor and Richardson, 1987) of the dideoxy termination method of Sanger et al. (1977).

Isolation of Genomic Cx37 DNA

The genomic mouse library (450,000 EMBL3 phages) was screened on 10 filters (132 mm, Hybond-N; Amersham International) with rat liver Cx26 cDNA (Zhang and Nicholson, 1989) labeled by random priming (2.4×10^5 cpm/ml; specific activity: 9.6×10^8 cpm/μg DNA) according to a standard protocol of plaque hybridization (Sambrook et al., 1989) under lowered stringency at 38°C, 50% formamide, and 5×SSC. 25 plaques were isolated after 2 rescreenings.

To determine which connexin genes were represented in these phages, 2 μl of lysate from each purified plaque was plated as a dot onto a lawn of *E. coli* LE392 on a BBL agarose (Seakem; FMC Bioproducts, Rockland, ME) plate and incubated overnight at 37°C. The next day a Hybond-N filter (Amersham International) was drawn and subjected to hybridization with various connexin cDNAs under stringent conditions (50% formamide, 42°C, 5×SSC). As control, 1 ng each of several connexin cDNAs (Cx26, Cx32, Cx37, or Cx43) was heat denatured in 15×SSC, spotted onto a Hybond filter, dried and fixed to the filter by UV irradiation at 254 nm. The control filter was hybridized under the same conditions as those containing immobilized DNA from recombinant phages. No cross reaction of Cx37 cDNA with Cx26, Cx32, or Cx43 cDNAs was found. Four purified plaques were identified that hybridized specifically to Cx37 cDNA. Phage DNA was isolated using standard protocols (Sambrook et al., 1989). Through comparison of the restriction maps of the Cx37 cDNA in conjunction with hybridization data on the digested phage DNA, it was concluded that one of the phage inserts contained the Cx37 coding sequence and ~11 kb of upstream flanking DNA. This phage DNA was double digested with EcoRI and SalI and the resulting insert fragments were subcloned into the plasmid pUC19. One of these EcoRI fragments of 2.9 kb contained the Cx37 coding sequence and ~1 kb 5′ flanking DNA. This DNA was sequenced by the chain modified termination method (Tabor and Richardson, 1987) using appropriate oligonucleotide primers derived from the Cx37 cDNA sequence. Computerized calculations of amino acid identities and alignments of different connexin sequences were performed by use of the Microgenie sequence analysis program (Beckman Instruments, Fullerton, CA).

Southern and Northern Blot Analysis

DNA from the liver of BALB/c mice was prepared according to a standard procedure (Sambrook et al., 1989). Total RNA from mouse tissues or exponentially growing cultured cells was isolated using the guanidinium isothiocyanate/cesium chloride method of Chirgwin et al. (1979). Equal amounts of total RNA from each tissue assayed were used for electrophoresis. The 1.5-kb cDNA fragment of Cx37 was labeled by the random priming procedure with α[^{32}P]dCTP (Amersham International) to a specific activity of 0.2–1 $\times 10^9$ cpm/μg DNA following the procedure recommended by the manufacturer. Southern and Northern blot hybridizations were carried out under stringent conditions (50% formamide, 42°C, 5xSSC) following a standard protocol (Sambrook et al., 1989). The amounts of polyA$^+$ RNA on Northern blots were standardized by hybridization to the 1.58-kb EcoRI–SalI fragment of mouse cytochrome *c* oxidase cDNA (clone pAG82; Herget et al., 1989) or the 1.2-kb cDNA of human glyceraldehyde 3 phosphate dehydrogenase (Hanauer and Mandel, 1984).

For comparison of the amounts of Cx37 and Cx26 transcripts, 20 μg of total RNA from each tissue was electrophoresed in 1.2% agarose in the presence of 2.2 M formamide (Sambrook et al., 1989). After ethidium bromide staining the RNA was blotted onto Hybond N membranes. Afterwards, no ethidium bromide–stained bands could be seen in the gel, suggesting that quantitative transfer had occurred. The membranes were cut into two halves at the position of a 2-kb RNA. For hybridization, the 1.1-kb cDNA fragment of rat liver Cx26 cDNA (clone 26-1; Zhang and Nicholson, 1989) and the 1.1-kb cDNA fragment (HincII–PstI) of mouse brain Cx37 cDNA (see above) were labeled with α[^{32}P]dCTP using the random priming reaction (sp act 5×10^8 cpm/μg DNA). For comparison of the hybridizations signals obtained with both probes, DNA dot blots with the corresponding plasmid DNAs were included in both hybridization experiments. Successive twofold dilutions containing 0.35–0.0014 fmol of either Cx26 cDNA (1.1 kb) in pGEM Blue vector (Promega Biotec, Madison, WI) or the Cx37 cDNA (1.5 kb) in pUC19 were applied to the dot blots. Both dot blots and corresponding Northern blots were prehybridized for 3 h at 42°C in a solution of 55% formamide, 1 M NaCl, 1% SDS, 10% dextran sulfate, and 100 μg/ml salmon sperm DNA and then hybridized for 50 h at 42°C using 25 ng of labeled Cx26 cDNA for that part of the filter containing RNA >2 kb and 25 μg/ng of labeled Cx37 cDNA for that part with RNA <2 kb. The filters were washed twice for 1 h with 2×SSC and 1% SDS at 56°C, and twice for 1 h with 0.2×SSC and 0.1% SDS at 60°C. They were exposed to Kodak XAR-5 x-ray film (Eastman Kodak Co., Rochester, NY) at −70°C using an intensifying screen. RNA signals on autoradiographs were standardized by comparison with cytochrome *c* oxidase transcripts (Herget et al., 1989) and quantified by densitometric evaluation and standardized on both halves of the Northern blot using the dot blot dilution series as an internal frame of reference.

Cell Lines and Culture Conditions

Mouse embryonic teratocarcinoma cells F9 (CRL 1720; American Type Culture Collection, Rockville, MD) (Bernstine et al., 1973) and rat fibro-

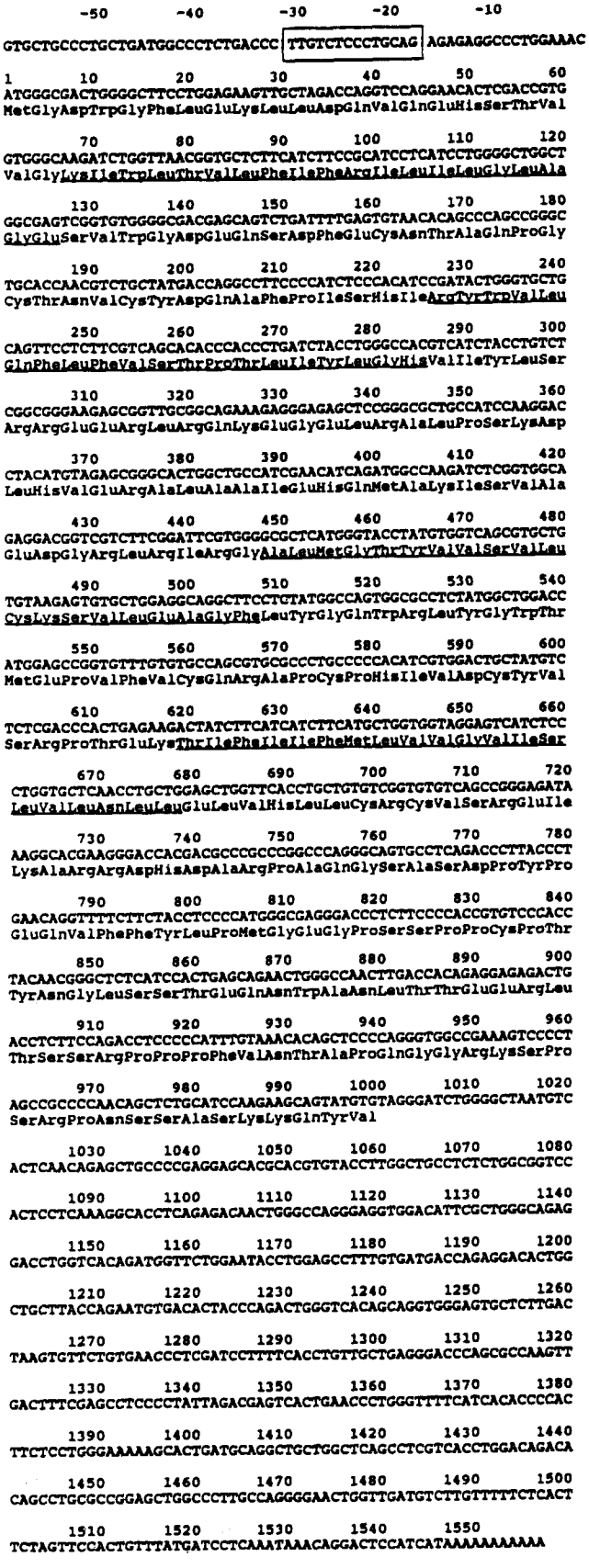

Figure 1. Nucleotide and amino acid sequences of mouse connexin 37. The cDNA sequence of mouse Cx37 was determined from nucleotides 70–1557. At position 485 a T residue in the cDNA sequence has been found instead of the A residue (shown) in the genomic sequence. This difference is probably an artifact of the blastic mammary carcinoma cells BICR-M1R-kd (Rajewsky and Grüneisen, 1972) were cultivated in DMEM (standard medium), supplemented with 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere of 10% CO_2 at 37°C. Mouse embryonic D3 stem cells (Doetschman et al., 1985) were maintained in the same medium with 15% FCS and 0.1 mM β-mercaptoethanol. Induction of F9 cell differentiation was performed with 10^{-7} M all-*trans* retinoic acid (Sigma Chemical Co.), 1 mM dibutyryl cAMP (Sigma Chemical Co.) or both components. Aggregated cells were cultivated in plastic dishes whose surface did not allow cultured cells to attach.

Preparation of Xenopus Oocytes

Adult female *Xenopus* frogs were anesthetized on ice and ovarian lobes were surgically removed and stored at 18°C in Leibovitz-15 (L-15) medium (Gibco Laboratories). L-15 was diluted 1:2 and buffered with 12.5 mM Hepes, pH 7.5. Antibiotics (streptomycin, penicillin, and gentamycin) were each added at a concentration of 10 μg/ml. Defolliculation was performed by manually dissecting the ovarian lobes into clumps containing ~50 oocytes and incubating them in Ca^{2+}-free OR-2 solution (83 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM Tris-buffer, pH 7.5) containing 2 mg/ml collagenase (Sigma Chemical Co.) for 1–1.5 h (Dascal et al., 1985).

Oocytes were then washed several times in OR-2 and placed back in L-15. Stage VI oocytes were selected and manually defolliculated with forceps. Defolliculated oocytes were injected with 40 nl of one of the following solutions: (*a*) diethyl thiopyrocarbonate-treated H_2O; (*b*) 0.2 ng/nl each of antisense oligodeoxynucleotides to *Xenopus* Cx38 (nucleotides 327–353) and cx43 (nucleotides 345–379) (numbering begins at the start of translation); (*c*) 0.2 ng/nl of mouse Cx37 cRNA coinjected with the previous two oligonucleotides. All nucleotides were dissolved in diethyl pyrocarbonate-treated water. Injected oocytes were incubated at 18°C for 48 h before devitellinization and pairing. Devitellinization was performed with forceps and oocytes were paired vegetal pole to vegetal pole in dishes with a thin layer of 1–2% agar coating the bottom. Recordings were done 24–36 h after pairing. Antisense oligodeoxynucleotides were prepared on an Applied Biosystems synthesizer (Applied Biosystems, Inc., Foster City, CA) with standard phosphoamidate chemistry. The cleaved, full-length products were then purified by HPLC (Beckman Instruments, Fullerton CA) on a MA7 plasmid column (Bio-Rad Laboratories, Oxnard, CA). Cx37 cRNA was prepared from a linearized plasmid template with T3 RNA polymerase in the presence of 5′ cap structure according to Promega instructions. Production of full-length transcripts was confirmed by agarose electrophoresis. Template DNA was prepared by insertion of the complete coding region of the Cx37 genomic clone (plus 17 bases of 5′ and 35 bases of 3′ untranslated flanking sequences) into the PstI/HincII sites of a bluescript SK^+ vector.

Electrophysiology

Junctional conductance was determined using the dual cell voltage-clamp technique described by Spray et al. (1981). Two modified Physiologic Instruments (Physiologic Instruments, San Diego, CA) voltage clamps (model-VCC600), made by Dr. Steve Thompson of the Veterans Administration Hospital of San Diego, were used to clamp both oocyte membrane potentials independently to −40 mV. Membrane potentials before clamping ranged from −40 to −65 mV. Electrodes (1.5 M KCl) of 1–3 MΩ resistance were used. Transjunctional potentials were produced by alternating depolarization and hyperpolarization of one of the paired oocytes in successive 5- or 10-mV steps of 30-s duration each. Recovery time of 1.5–2 min was allowed between voltage steps. Current and voltage were recorded on a brush chart recorder (model 2400; Gould Inc., Glen Burnie, MD). In addition, some traces were recorded on tape via an Instrutech converter (VR100 PCM A/D) (Elmont, NY) using a high frequency cutoff of 30–100 Hz. Current resolution after the initial capacitative transient after a voltage step was ~20 ms.

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cDNA synthesis (see Results). The 5′ end of the coding sequence has been completed by sequence data from the corresponding mouse genomic clone. The presence of a potential splice acceptor site (*boxed*) and lariat formation sequence (at −51 to −45) indicates that not all of the 5′ untranslated sequence shown may be present in mature Cx37 mRNA. Four amino acid sequences that are assumed to form transmembrane regions are underlined. These sequence data are available from EMBL/GenBank/DDBJ under accession number X57971.

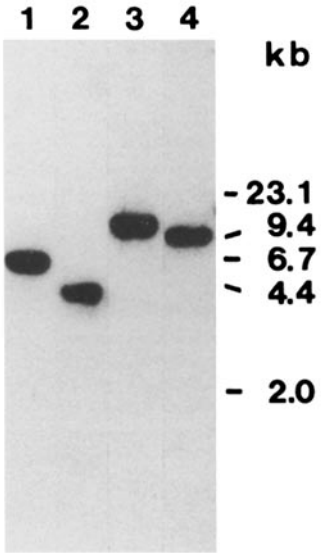

Figure 2. Southern blot hybridization of mouse Cx37 cDNA to mouse genomic DNA. Mouse genomic DNA was digested with HindIII, HincII, EcoRI, and BamHI, electrophoresed, and subjected to Southern blot hybridization using mouse Cx37 cDNA following a standard protocol (Sambrook et al., 1989). The autoradiograph obtained after 7 d of exposure is shown.

Results

Isolation of Cx37 cDNA and Genomic Clones

From a reduced stringency screen (38°C, 50% formamide, 5×SSC) of a mouse cDNA library with a Cx32 rat liver cDNA clone (Heynkes et al., 1986) one clone containing a unique 1.5-kb insert was isolated. Sequencing revealed a novel member of the connexin family (Fig. 1). While the 3′ terminus contained the expected polyA region preceded 20 nucleotides upstream by a consensus polyadenylation signal (A_2TA_3), the cDNA appeared incomplete at the 5′ end. Alignments with other connexin sequences (Beyer et al., 1987; for review see Willecke and Traub, 1990) suggested that 69 nucleotides of coding sequence and an undetermined length of 5′ untranslated material were missing from this cDNA clone. To complete the coding sequence this partial cDNA was used in a secondary screen of mouse genomic clones originally selected by low stringency screening with a rat cx26 cDNA probe. One phage was selected which, after subcloning into pUC19, yielded a 2.9-kb EcoRI/SalI fragment. This clone was determined to contain the complete coding sequence in addition to ~1 kb of upstream untranslated material.

Sequence Characterization of Mouse Cx37 Genomic and cDNA Sequences

Southern blot hybridization of Cx37 cDNA to mouse genomic DNA digested with different restriction endonucleases (Fig. 2) suggested that a single copy of Cx37 exists in the mouse genome. Under these conditions, single fragments of ~7, 4, 13, 9 kb were obtained after treatment with HindIII, HincII, EcoRI, and BamHI, respectively. The human gene coding for Cx37 has recently been mapped to chromosome 1 in the human genome (Willecke et al., 1990*b*). As has proven to be the case in two other connexin genes (Cx32, Miller et al., 1988; Cx43, Fishman et al., 1990) the coding region is uninterrupted by introns but an upstream intron is suggested by the location of a consensus splice acceptor site at position −17 upstream of the ATG translational start codon. This is preceded by a putative lariat-forming sequence at −51 to −45 of the genomic Cx37 sequence shown in Fig. 1. The sequence of the Cx37 cDNA was identical to the genomic clone with the exception of position 485 (see Fig. 1) where a T residue was found in the cDNA sequence instead of an A in the genomic clone. This difference was confirmed in several sequencing experiments. Thus the genomic sequence codes for a lysine, whereas the cDNA clone codes for a methionine residue at position 162 of the Cx37 amino acid sequence. We think that the T residue in the cDNA clone is an artifact of the cDNA synthesis. All connexin sequences determined so far (including five new mouse connexin sequences characterized in our laboratory) show a lysine or arginine residue at this position of the amino acid sequence that is part of the putative transmembrane region 3 (see next paragraph).

Comparison of the Cx37 Sequence with Other Connexin Sequences

The 333 amino acids of the predicted coding sequence add up to a relative molecular mass of 37,600 D. We designate this connexin as mouse Cx37 or Cx37.6. The predicted Cx37 sequence fits the pattern established directly for other members of the connexin family by binding site-specific antibodies and by membrane protection from proteolysis (Zimmer et al., 1987; Milks et al., 1988; Goodenough et al., 1988; Hertzberg et al., 1988; Yancey et al., 1989). We analyzed the Cx37 amino acid sequence according to Rao and Argos (1986) for the tendency to form transmembrane regions. Four potential transmembrane helices of 20–25 residues

putative topological region of Cx37	% amino acid identities to Cx43	to Cx32	to Cx38
cytoplasmic region A:	64 (64)	45 (55)	59 (68)
transmembrane region 1:	75 (90)	55 (85)	80 (90)
extracellular region B:	76 (82)	73 (76)	88 (91)
transmembrane region 2:	70 (85)	50 (65)	95 (95)
cytoplasmic region C:	37 (57)	28 (35)	39 (52)
transmembrane region 3:	45 (65)	45 (65)	60 (70)
extracellular region D:	57 (76)	51 (68)	62 (76)
transmembrane region 4:	70 (90)	40 (65)	80 (95)
cytoplasmic region E:	23 (30)	15 (23)	30 (38)

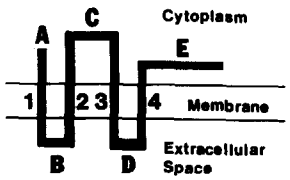

Figure 3. Amino acid identities of the putative topological domains of mouse Cx37 compared to rat Cx43, rat Cx32, and Xenopus Cx38. The values in brackets include amino acids which are not identical but have similar properties, e.g., Lys versus Arg. For better comparison, the subdivision of the Cx37 and Cx43 amino acid sequences into putative domains was carried out by analogy to the established topological models of Cx32 (Milks et al., 1988), and Cx43 (Yancey et al., 1989). Numbers refer to transmembrane regions and letters to cytoplasmic and extracellular regions illustrated schematically.

long were obtained, with the third showing a marked amphipathic character. The connecting loops between the first and the second, and third and fourth hydrophobic domains, shown to be extracellular in Cx32 (Milks et al., 1988; Goodenough et al., 1988), Cx43 (Yancey et al., 1989), and Cx26 (Zhang, J. T., and B. J. Nicholson, manuscript submitted for publication), each have the conserved pattern of three cysteine residues. The specific pattern of amino acid sequence identities between mouse Cx37, rat Cx32, rat Cx43, and *Xenopus* Cx38 is shown in Fig. 3. In this case the subdivision of Cx32 and Cx43 into putative topological regions was in accordance with previous analyses (Milks et al., 1988; Beyer et al., 1989; Hertzberg et al., 1988; Yancey et al., 1989). The comparison shows clearly that, of the mammalian connexins mouse Cx37 is more homologous to rat Cx43 (59% amino acid identity overall) than to rat Cx32 (47%), although the highest level of amino acid identity detected was to Xenopus Cx38 (66%). For evaluation of overall identities only amino acid sequences from the NH_2 terminus to the end of the fourth putative transmembrane region were taken into account. In particular the putative transmembrane regions 1, 2, and 4 of Cx37 show >80% amino acid identity to *Xenopus* Cx38, >70% identity to Cx43 but <55% to Cx32. In contrast, putative transmembrane region 3 of Cx37 shows 60% amino acid identity to Cx38 and only 45% identity to both Cx43 and Cx32. As with other connexins, domains shown to be cytoplasmic, particularly regions C and E of Fig. 3, show very little or no significant sequence similarity within the gene family.

The putative transmembrane domain 3 of Cx37 retains the amphipathic character (when modeled as an α-helix) seen in other connexins. The conserved lysine residue in this helix (replaced by an arginine residue in several other connexins cf. Gimlich et al., 1990) is preceded by a cysteine in Cx37, in contrast to the phenylalanine seen in other connexins. The other conserved charged residue in this "helix", glutamate 165, is also preserved in Cx37, but is preceded by a leucine rather than the phenylalanine found in most other connexins sequenced to date.

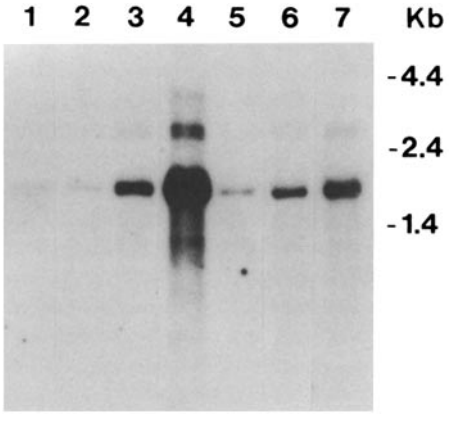

Figure 4. Autoradiograph of a Northern blot after hybridization with a labeled Cx37 cDNA probe. Each lane contains 20 μg of total RNA isolated from the following mouse tissues: adult intestine (lane *1*), adult skin (lane *2*), adult heart (lane *3*), adult lung (lane *4*), adult spleen (lane *5*), embryonic skin (lane *6*), and embryonic kidney (lane *7*).

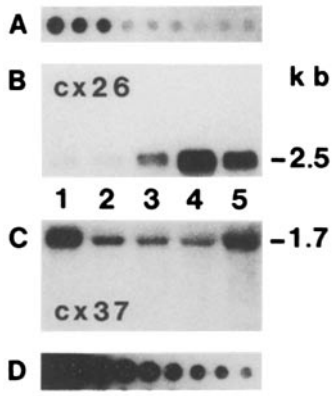

Figure 5. Comparison of the relative levels of transcripts for Cx26 and Cx37 in several mouse tissues. 20 μg of total cellular RNA from embryonic brain (lane *1*), adult brain (lane *2*), embryonic liver (lane *3*), adult liver (lane *4*), and adult kidney (lane *5*) were separated electrophoretically and transferred onto a nylon membrane. The membrane was then cut at the position of a 2-kb RNA marker so as to separate Cx26 (2.5 kb) and Cx37 (1.7) messages. The appropriate half was hybridized to a 1.1-kb fragment of Cx26 (*B*) or Cx37 (*C*) cDNA. To standardize the hybridization signals obtained with each probe, a dot blot with serial dilutions of the respective plasmid DNAs was included in both hybridization experiments (*A*: Cx26; *D*: Cx37). Further details of the experiment are described under Results.

In addition to these structural features relating to the topology, the amino acid sequence of Cx37 also has several consensus motifs for posttranslational modifications in the putative cytoplasmic regions. For example, at position 299–304 the typical amino acid sequence (Arg-X-X-Ser-X-Arg) for phosphorylation by protein kinase C is found (see Kemp and Pearson, 1990). Furthermore, there is a consensus motif for phosphorylation by the cell cycle–dependent kinase cdc28 at position 318–320 (Lys-Ser/Thr-Pro). Two possible *N*-glycosylation sequences (Asn-X-Ser/Thr) are also found at position 293–295 and 324–326. Their probable cytoplasmic disposition, however, suggests that they are not utilized. A possible binding site for calmodulin (consisting of an amphipathic helix with positively charged surface [O'Neil and DeGrado, 1990]) is located at the position 228–243.

Expression of Cx37 mRNA

In Figs. 4 and 5 *C* the expression of Cx37 mRNA in several mouse tissues was studied by Northern blot hybridization. In total cellular RNA of all tissues investigated an mRNA of 1.7 kb hybridizes to the Cx37 cDNA under higher stringency (42°C, 50% formamide, 5×SSC). By quantitative densitometric evaluation of an autoradiograph after Northern blot hybridization (Fig. 4) (and additional lanes of the same Northern blot, not shown) the relative amounts of Cx37 transcripts were normalized to the amounts of cytochrome *c* oxidase transcripts and estimated as: adult lung (100%), embryonic brain (4.1%), embryonic kidney (3.2%), adult spleen (2.5%), embryonic skin (2.6%), adult liver (1.7%), embryonic liver (1.3%), adult intestine (1%), adult skin (0.9%), adult heart (0.9%), adult kidney (0.7%), and adult brain (0.4%). How these RNA levels for Cx37 relate to those of other connexins was established through quantitative comparisons with a representative member of the family, Cx26, in liver, brain, and kidney. A Northern blot of total RNA from these tissues (Fig. 5) was cut to separate the Cx37 (1.7 kb) as well as Cx26 (2.5 kb) mRNAs, and hybridized to a 1.1-kb DNA probe of the corresponding connexin gene (Fig. 5, *B* and *C*). For both halves of the Northern blot, a

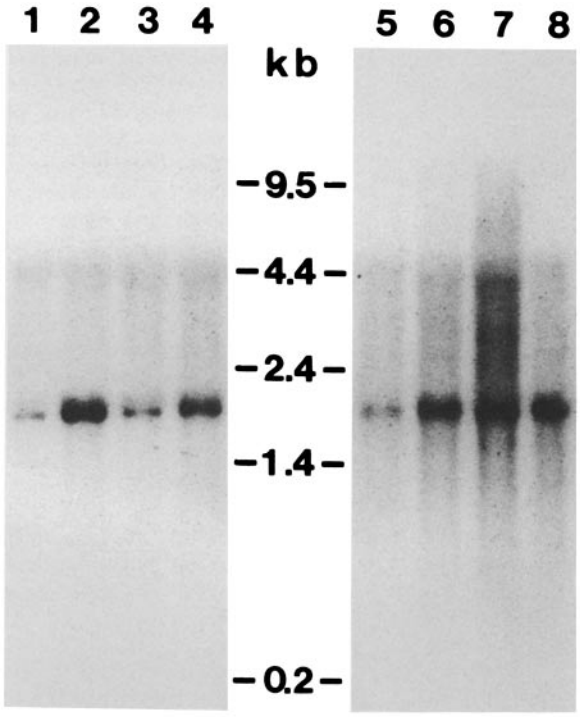

Figure 6. Expression of Cx37 mRNA in mouse embryonic carcinoma F9 cells. The cells were cultivated as attached cells for 4 d (lanes *1–4*) or as aggregates for 7 d (lanes *5–8*) in standard medium without further additives (lanes *1* and *5*) or in the presence of 10^{-7} M retinoic acid (lanes *2* and *6*), 10^{-3} M dibutyryl-cAMP (lanes *3* and *7*), or both components (lanes *4* and *8*). 20 μg of total RNA from cells of each culture were hybridized at high stringency to the Cx37 cDNA.

dilution series of the corresponding plasmid DNA was included to normalize the signal from each probe (Fig. 5, *A* and *D*). After correcting for the relative strength of signals from the two probes (~16-fold higher for Cx37) we estimated the following ratios of Cx26 to Cx37 mRNA: embryonic brain: 3; adult brain: 12; embryonic liver: 75; adult liver: 220; and adult kidney: 36.

Relatively weak expression of rat Cx37 mRNA (1.7 kb) was found in the endometrium and myometrium of rat uterus during pregnancy at decidualization and at term (Winterhager et al., 1991). Furthermore, the Cx37 transcript was found in BICR cells (rat mammary carcinoma cell line), D3 mouse embryonic stem cells, and F9 cells (mouse embryonic carcinoma cell line). The latter cells differentiate in culture in the presence of retinoic acid and dibutyryl cAMP to parietal endoderm cells when attached to substrate but form visceral endoderm in response to retinoic acid when growing in suspension (Strickland and Mahdavi, 1978; Strickland et al., 1980; Hogan and Taylor, 1981). Both tissues represent differentiations of the inner cell mass of mouse embryos. Relative to nontreated cells, we found a two to threefold increase of Cx37 transcripts in response to retinoic acid in both F9 parietal and visceral endoderm cells (Fig. 6). Dibutyryl cAMP caused no change in Cx37 transcripts in attached F9 cells but led to a twofold increase in Cx37 mRNA in F9 cells growing in suspension (Fig. 6). The regulation of Cx37 transcripts in F9 endoderm cells by retinoic acid is similar to the one observed for Cx43 mRNA under the same experimental conditions (Willecke et al., 1990*a*).

Functional Expression of Mouse Cx37 in Xenopus Oocytes

The complete coding sequence of Cx37 derived from the genomic clone was subcloned into a bluescript SK^+ vector (see Materials and Methods). Capped Cx37 RNA was synthesized in vitro using T3 RNA polymerase, injected into *Xenopus* oocytes which were subsequently stripped of their vitelline envelopes, and paired. The background of endogenous coupling in this system, contributed mainly by *Xenopus* Cx38 (Ebihara et al., 1989), and possibly to some extent by *Xenopus* Cx43 (Gimlich et al., 1990), could be eliminated by coinjection of antisense oligodeoxynucleotides to these sequences (specifically Cx38 (nucleotides 327–353) and Cx43 (nucleotides 345–379): in each case, numbering of the nucleotide sequence starts at the beginning of transcription). This presumably occurs through RNase H digestion of the mRNA at the point of hybridization and subsequent breakdown of the mRNA (Dash et al., 1987). While both oligonucleotides were used in some experiments, we generally found that the antisense Cx38 construct was sufficient to eliminate all endogenous coupling after a 48-h incubation. Since the oligonucleotide sequences were chosen to avoid crosshybridization between connexin coding sequences, this result strongly suggests that *Xenopus* Cx43 mRNA is not efficiently translated in stage VI oocytes.

While oocyte pairs injected with antisense oligonucleotides to *Xenopus* Cx38 alone had consistently undetectable levels of coupling (<2 nS), those coinjected with antisense oligonucleotide and Cx37 cRNA display high levels of coupling ranging from 70 nS to 2 μS in 13 pairs tested (mean: 930 μS). In some cases where endogenous coupling was minimal even in the absence of antisense oligonucleotides, Cx37 mRNA alone induced qualitatively and quantitatively identical results to the coinjected oocytes. Thus, as would be expected from lack of homology between mouse Cx37 and *Xenopus* Cx38 cDNAs in the region to which the oligonucleotide was made, the antisense oligonucleotides do not affect exogenous channel activity.

Quantitative analysis of junctional currents induced by Cx37 showed that initial junctional conductance (g_j(i)), measured within 20 msec of the onset of the voltage step, decreased with increasing positive or negative transjunctional voltages (V_j) (Fig. 7 *B*). This very fast voltage gating of the channels can be described by a single Boltzmann relationship with an A (cooperativity constant) of 0.1 and a V_o (voltage for half maximal drop in conductance) of ± 60 mV. Conductance dropped maximally to ~40% of the initial levels. This fast voltage sensitivity, although seen to a very minor extent in Cx32 expressing oocytes (<10% drop in g_j at V_j's of ± 100 mV; personal observations) has not been observed in intercellular coupling mediated by other connexins. A kinetically slower sensitivity to V_j typical of most gap junctions analyzed to date (Spray et al., 1981; Ebihara et al., 1989; Barrio et al., 1991) is also observed with steady state conductance levels (g_j(ss)) measured after the transjunctional voltage step had been maintained for 30 s (Fig. 7 *B*). The sensitivity of this slow response to V_j was higher than that recorded for other connexins in oocytes (Swenson et al., 1989; Werner et al., 1989) and more comparable to that seen

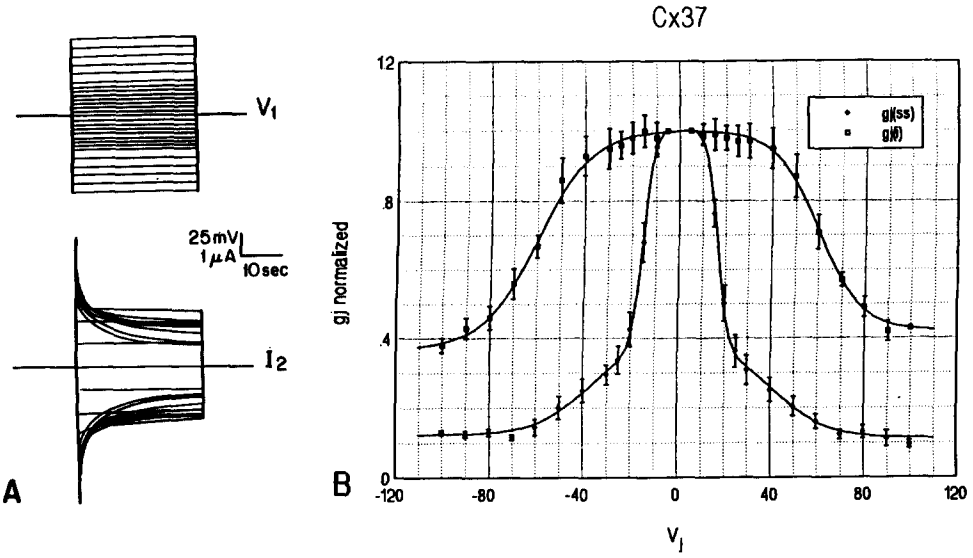

Figure 7. Junctional conductance (g_j) of Cx37 induced coupling between *Xenopus* oocytes as a function of transjunctional voltage. (*A*) Representative traces from an experiment using dual cell voltage clamps of paired oocytes injected with Cx37 cRNA show the decay in g_j in response to steps in V_j. In response to voltage steps in cell 1 of a pair (V_j), current across the junctions could be measured as the change in current in cell 2 (I_2) necessary to maintain a constant −40 mV holding potential in this cell. (*B*) A plot of g_j (normalized with respect to the initial conductance measured at ±10 mV) versus V_j for Cx37 cRNA injected oocyte pairs. Both cells are initially clamped to −40 mV. Both initial g_j (●) obtained by extrapolating exponential current decays back to 0 time, and steady state g_j (⊡), measured 30 s after the onset of the voltage step, show a dependence on V_j. Curves are computer fits to either a single Boltzmann decay (initial conductance) or two superimposed Boltzmann decays (steady-state conductance). Parameters are given in the text.

in *Fundulus* blastomeres (Spray et al., 1981). The gating of these cx37 channels appears to be complex both kinetically and in terms of V_j sensitivity. The decay of junctional current (I_2 in Fig. 7 *A*) is not fit by a simple exponential, but appears to have two components. In addition, the plot of g_j (ss) versus V_j (Fig. 7 *B*) is not described by a simple Boltzmann function, but can be modeled as two superimposed gates described by Boltzmann parameters of $V_o = \pm 16$ mV, $A = 0.44$ for the more sensitive element, and $V_o = \pm 40$ *mV*, $A = 0.09$ for the less sensitive component. These models were obtained after subtracting the effect of the initial conductance decay (g_j(i)) with respect to V_j. Given the absence of any coupling by endogenous connexin channels in antisense oligonucleotide-injected oocytes paired either together or with Cx37 injected oocytes, we conclude that the observed responses are properties of the Cx37 channel itself.

Discussion

The coding sequence of mouse Cx37 DNA was determined by comparison of a partial cDNA from adult mouse brain with its corresponding genomic clone. The first 69 nucleotides at the 5′ end of the coding sequence were missing from the cDNA clone. As in other connexin genes characterized to date (Miller et al., 1988; Fishman et al., 1990), Cx37 has no introns in its coding region. The complete coding sequence plus the 3′ nontranslated region of the cx37 cDNA including a poly A tail is shorter (1,557 bp) than the mRNA detected on Northern blots (~1.7 kb). However, we cannot assume that the 5′ untranslated region seen in the genomic clone is that found in the mRNA, particularly since an intron has been demonstrated or deduced to occur 16 or 17 bases upstream of the initiator ATG in three other connexins (Cx32: Miller et al., 1988; Cx43: Fishman et al., 1990; and Cx26: Zhang and Nicholson, 1988; Hennemann, H., G. Kozjek, E. Dahl, B. Nicholson, and K. Willecke, manuscript in preparation). The location of consensus splice acceptor and lariat formation sites in the Cx37 genomic sequence (Fig. 1) is consistent with a similar intron location in this gene. Given the indirect manner of infering intron boundaries from genomic clones alone, final determination of the 5′ untranslated sequence of the Cx37 RNA must await isolation of a full-length cDNA.

Of the other mammalian connexins published to date, Cx37 is most similar to cx43 (i.e., by the classification of Risek et al., 1990, it would belong in the "α class" of connexins).

Overall amino acid identity with Cx43 is 59% (excluding putative cytoplasmic COOH-terminal region), but an even closer homology is evident in comparison of mouse Cx37 with *Xenopus* Cx38 (66% amino acid identity). Overall homology levels are limited by the highly divergent COOH-terminal tail and the loop connecting putative transmembrane domains 2 and 3. The lack of significant sequence homology in these proposed cytoplasmic domains suggests that mouse Cx37 is not an analogue of *Xenopus* Cx38 (compare Cx43 sequence in human (Fishman et al., 1990), rat (Beyer et al., 1987), and *Xenopus* (Gimlich et al., 1990)). This conclusion is supported by the relatively wide distribution of Cx37 message in the adult mouse, albeit at low levels, compared to the restricted expression of Cx38 transcripts in *Xenopus* to the oocytes through blastocyst stages of embryogenesis and the adult ovary. Channel properties of these connexins also differ markedly with respect to voltage sensitivity when expressed in oocytes. Thus it seems that Cx37 represents a novel member of the connexin gene family.

Expression of Cx37 cRNA in *Xenopus* oocytes demonstrates that it is competent to form intercellular channels. It is likely that Cx37 directly forms the structure of the channels themselves rather than serving to indirectly induce endogenous structures. This can be deduced not only from its homology to other connexins which have been demonstrated to be structural components of intercellular channels (Cx32: Hertzberg and Gilula, 1979; Henderson et al., 1979; Spray et al., 1986; Young et al., 1987) but also from its capacity to determine specific channel properties such as voltage gat-

ing (known to be an integral property of the channel in other systems) which differ substantially from those of endogenous oocyte channels. In this context, it is of interest to consider the model of channel closure proposed by Unwin (1989) in which the phenylalanine residues located adjacent to the hydrophilic surface of transmembrane helix 3 in virtually all connexins might rotate into the aqueous pore causing it to collapse. In such a model, the substitution of two of the three phenylalanines with smaller residues (cysteine 161 and leucine 165 in Cx37) might be expected to affect gating kinetics through either destabilization of the open state (by van der Waals interactions), or reduction of the energy of the transition state between open and closed conformations (by reduced steric hindrance during rotation).

We do not know why Cx37 transcripts are highly expressed in lung. Future studies using in situ hybridizations will be needed to show in which cell type the Cx37 transcripts are located. Several years ago it was shown by freeze fracture analysis (Bartels, 1979) that human interstitial cells (myofibroblasts) of the pulmonary interalveolar septa are connected by gap junctions. Regarding other connexins in this tissue, Zhang and Nicholson (1989) found no Cx26 transcripts and relatively low levels of Cx32 in rat. We have recently detected Cx43 in immunoblots of mouse lung plasma membranes (Traub, O., and T. May, unpublished result).

Cx37 mRNA is expressed in several tissues and cell lines that also show high expression of Cx43 mRNA. While the distribution of either connexin by cell type in these tissues needs to be determined, the possibility exists for interactions between the two proteins within a given gap junctional channel, analogous to that already demonstrated in the oocyte expression system between Cx43 and Cx38 (Swenson et al., 1989, and Werner et al., 1989) or Cx32 and Cx26 (Barrio et al., 1991). As has proven to be the case in the latter example, the heteromeric channels can have properties that are distinct from those of either homomeric channels. Recently we have found (unpublished results) that the mRNA of a new mouse connexin (termed connexin40) is also highly abundant in lung and shows a pattern of tissue expression that is very similar to the one reported in this paper for Cx37 transcripts. We speculate that this new connexin40 may be another candidate for interaction with Cx37 in heteromeric gap junction channels. Clearly, the potential interaction of major and minor connexin components in all tissues and their effects on coupling characteristics need to be carefully assessed as the complexity of this protein family increases.

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