The murine gap junction gene connexin36 is highly expressed in mouse retina and regulated during brain development

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Abstract A new gap junction gene isolated from rat brain cDNA, mouse retina cDNA and mouse genomic DNA is called connexin36, since it codes for a connexin protein of 321 amino acids corresponding to the theoretical molecular mass of 36 045 kDa (rat) and 36 084 kDa (mouse). Only one amino acid residue differs between rat and mouse connexin36. In the single murine connexin36 gene, an 1.14-kb intron interrupts the coding region, similar as in the homologous skate connexin35 gene. Because of this unique feature, mouse connexin36 differs from the other 13 murine connexin genes and is suggested to form a new δ subclass of connexins. Connexin36 mRNA (2.9 kb) is highly expressed in adult retina and less abundant in brain where it gradually increased during fetal development until day 7 post partum, and decreased thereafter.

Key words: Rat connexin36 cDNA; Mouse connexin36 gene; Connexin δ subgroup; Retinal connexin

1. Introduction

Gap junctions are arrays of intercellular channels that are formed by docking of two hemichannels comprised of connexin (Cx) protein subunits. These channels allow direct intercellular diffusion of ions, metabolites and second messenger molecules [1,2]. So far, 13 different connexin genes have been described in the murine genome (cf. [3], [4–6]). Connexin genes are expressed in a cell type-specific manner with overlapping expression patterns. Gap junctional intercellular communication has been suggested to be involved in metabolic cooperation, cellular synchronization, growth control and regulation of development (cf. [1,2,7]).

It has been reported that expression of connexins in the brain is differentially regulated during pre- and postnatal development. For example, Cx26 is downregulated around birth, whereas Cx32 and Cx43 proteins are upregulated postnatally [8]. Cx26 is expressed in prenatal neurons, Cx32 in oligodendrocytes and neurons and Cx43 is abundantly expressed in astrocytes [8]. More recently it has been shown that Cx32 and Cx45 are coexpressed in oligodendrocytes [9,10]. Cx30 mRNA is highly upregulated in the brain 4 weeks after birth [6]. Recently, expression of connexin genes in the nervous system has been reviewed [11].

Although electrophysiological measurements and microinjection of tracer molecules have revealed extensive gap junctional communication among neuronal cells in the retina of different species (cf. [12]), few studies have aimed to identify the cell type specific expression of connexin genes in the retina (cf. [13]).

Recently O'Brien et al. [14] have cloned Cx35 cDNA from skate retina where it is exclusively expressed. This new skate connexin gene was different from all other known connexin genes in that it contained an intron in the coding sequence. Using degenerated primer molecules, deduced from skate Cx35 cDNA we searched for homologous connexin sequences expressed in murine retina and brain. We found a new connexin gene, highly homologous in mouse and rat, that was designated Cx36 in rat and mouse based on the theoretical molecular mass of its deduced amino acid sequence.

When we prepared our results for publication, Condorelli et al. [15] very recently reported the sequence of mouse Cx36 and its expression in rat brain neurons. Here we describe additional relevant characteristics of the murine Cx36 gene, not reported in [15]. The single mouse connexin gene is expressed about 18-fold higher in adult retina than in brain and it is upregulated in mouse brain before birth until day 7 post partum. Based on its unique genomic structure, Cx36 is suggested to form a new δ subclass of murine connexin genes.

2. Material and methods

2.1. RT-PCR analysis and cloning of mouse retina Cx36 cDNA

Total RNA from mouse retina was isolated with the TRIzol-Reagent according to the manufacturer (Gibco-BRL, Eggenstein, Germany). Aliquots of 2 μg RNA were incubated with 1 μg oligo(dT)15 primer (Promega, Madison, WI, USA) for 10 min at 68°C in a total volume of 20 μl and chilled on ice briefly. After primer annealing, poly(A)$^+$ RNA was reverse transcribed using 5 units AMV reverse transcriptase (Promega). The RT buffer consisted of 50 mM Tris-HCl (pH 8.3), 40 mM KCl, 6 mM $MgCl_2$, 10 mM dithiothreitol, 40 units of RNAsin ribonuclease inhibitor (Promega) and 125 μM of each dNTP (Promega). Reactions (50 μl total volume) were incubated for 60 min at 42°C and thereafter for 5 min at 95°C. The cDNA preparations were stored at −70°C.

Aliquots of the transcribed cDNA (1/25, approximately 0.1 ng) were amplified using the following degenerated primers which were derived from the sequence of skate Cx35 [14]: upstream primer Cx35USP2 5′-ATG TTT GTA TGC AAC ACC CTC CAG CCC GGC TG-3′ and downstream primer Cx35DSP3 5′-CAG AGT CGC TGG ACT GAG TTC GCC CGA AGT TG-3′. Reaction mixtures (50 μl) contained 20 mM Tris-HCl (pH 8.4), 250 μM dNTPs, 1.25 mM $MgCl_2$, 50 mM KCl, 2 μM of each primer and 1.5 units Ampli Taq DNA-Polymerase (Perkin Elmer, Foster City, CA, USA). PCR was carried out for 40 cycles using a PTC-100 Thermal Cycler (MJ Research, Watertown, MA, USA) with the following program: denaturing at 94°C for 1 min, annealing at 60°C for 1 min, elongation at 72°C for 2 min. After gel electrophoresis in an 1% agarose gel, an 802-bp fragment was excised, purified by the gel extraction method of Qiagen (Hilden, Germany), cloned into the pGEM-T Vector (Promega) and commercially sequenced by MWG BioTech, Ebersberg, Germany.

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Abbreviations: RT-PCR, reverse transcription polymerase chain reaction; Cx, connexin; GAPDH, glyceraldehydephosphate dehydrogenase; UTR, untranslated region

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2.2. Cloning of mouse genomic Cx36 DNA and rat brain Cx36 cDNA

Approximately 8×10^6 plaque forming units of a mouse 129 Sv λFixII genomic library (Stratagene, La Jolla, CA, USA) and approximately 6×10^6 plaque forming units of a fetal rat brain λgt11 cDNA library (Clontech, Palo Alto, CA, USA) were plated on Petri dishes, transferred onto Hybond-N$^+$ filters (Amersham International, Amersham, Bucks, UK) according to instructions of the manufacturer and prehybridized for 6–9 h at 42°C in 50% formamide, 5×SSC, 0.5% sodium dodecyl sulfate (SDS), 5×Denhardt's solution with 150 μg/ml heat denatured, sheared salmon sperm DNA (Boehringer Mannheim, Germany).

The 802-bp RT-PCR fragment of mouse Cx36 exon 2, previously cloned from mouse retina total RNA, was ^{32}P-labelled using the random primed labelling method (multiprime labelling Kit, Amersham) to a specific activity of $0.5–1.0\times10^9$ cpm/μg DNA and added to fresh prehybridization solution at 5×10^5 cpm/ml. Hybridization at high stringency was carried out for 12–24 h at 42°C. The filters were washed twice for 20 min in 1×SSC/0.1% SDS at 65°C and finally in 0.1×SSC/0.1% SDS at 65°C and exposed to XAR X-ray film (Eastman Kodak, Rochester, NY, USA) with intensifying screen at −70°C.

Six recombinant Cx36 genomic and three cDNA containing phage clones were isolated by several rounds of rescreening under the same conditions. DNA of one of these genomic and cDNA phage clones was isolated using standard protocols [16].

A Cx36 rat cDNA *Eco*RI fragment was excised from a λgt11 cDNA phage and a Cx36 genomic *Not*I fragment (10.5 kb) was excised from a λFixII genomic phage. Both fragments were subcloned in pBluescript-SKII$^+$ (Stratagene) and sequenced by MWG BioTech.

2.3. Southern blot analysis

Genomic DNA was isolated from mouse 129 Sv embryonic stem cells according to [16]. Ten-μg aliquots were digested with 40 units each of the following restriction enzymes: *Apa*I, *Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII, *Kpn*I, *Pvu*II, *Xba*I, *Sal*I, *Pst*I (Boehringer Mannheim). These digests were separated in a 0.8% agarose gel and subsequently transferred to Hybond-N$^+$ membrane (Amersham) by capillary diffusion in 0.4 M NaOH after depurination with 0.25 N HCl for 10 min. The blot was probed with the 802-bp RT-PCR fragment of mouse Cx36 exon 2, which had been ^{32}P-labelled using the random primed labelling method (Amersham) described above.

Hybridization was carried out under conditions of high stringency (50% formamide, 5×SSC, 5×Denhardt's solution and 0.5% SDS) in the presence of 50 μg/ml heat-denatured salmon sperm DNA. After the last washing step with 1×SSC/0.1% SDS for 5 min at 65°C, the nylon membrane was sealed and exposed to Kodak XAR X-ray film with intensifying screen at −70°C for 14 days.

2.4. Northern blot analysis

Total RNA (20 μg) from 13 adult mouse (CD1) tissues and seven developmental stages of the brain (day 14 post coitum to postnatal day 14) was prepared with TRIzol-reagent according to the manufacturer (Gibco-BRL). RNA was electrophoresed [16] and transferred to Hybond-N nylon membrane (Amersham) by capillary diffusion in 20×SSC. Transcript sizes were determined by comparison to an RNA molecular weight ladder (Gibco-BRL). The blot was probed with the ^{32}P-labelled 0.8-kb RT-PCR fragment of mouse Cx36 exon 2. Hybridization was carried out under high stringency conditions (55% formamide, 5×SSC, 5×Denhardt's and 0.5% SDS) in the presence of 60 μg/ml heat-denatured salmon sperm DNA for 12–24 h at 42°C [16]. After the last washing step with 0.1×SSC/0.1% SDS for 10 min at 65°C, the nylon membranes were sealed in plastic wrap and exposed to Kodak XAR X-ray film with intensifying screens at −70°C for two to three weeks. The amounts of total RNA on the Northern blot were standardized by hybridization to a probe of glyceraldehyde-phosphate dehydrogenase (GAPDH) [17]. The densitometric analysis was carried out with the Scan Package (Version 4.0), Biometra, Göttingen, Germany.

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ttgcttctttaattgggggcaattagctccctgccaggaggtgcggcccggggaggggcc
aggaccaagaacgtgcccggtactgcccagtctttgtctgctgcctccggatgcacagcg

1          10         20         30         40         50         60
ATGGGGGAATGGACCATCTTGGAGAGGCTGCTGGAAGCCGCGGTGCAGCAGCACTCCACT
 M  G  E  W  T  I  L  E  R  L  L  E  A  A  V  Q  Q  H  S  T

          70         80         90         100        110        120
ATGATTGGGAGGATCCTGTTGACTGTGGTGGTGATCTTCCGGATACTCATTGTGGCCATT
 M  I  G  R  I  L  L  T  V  V  V  I  F  R  I  L  I  V  A  I

         130        140        150        160        170        180
GTAGGGGAGACGGTGTACGATGATGAGCAGACCATGTTTGTGTGCAACACCCTGCAGCCC
 V  G  E  T  V  Y  D  D  E  Q  T  M  F  V  C  N  T  L  Q  P

         190        200        210        220        230        240
GGCTGTAACCAGGCCTGCTATGACCGCGCCTTTCCCATCTCCCATATACGTTACTGGGTC
 G  C  N  Q  A  C  Y  D  R  A  F  P  I  S  H  I  R  Y  W  V

         250        260        270        280        290        300
TTCCAGATCATAATGGTGTGCACCCCCAGTCTCTGTTTCATCACCTATTCTGTGCATCAA
 F  Q  I  I  M  V  C  T  P  S  L  C  F  I  T  Y  S  V  H  Q

         310        320        330        340        350        360
TCTGCCAAGCAGAGAAGCGCCGGTACTCGACTGTCTTCCTAGCCCTGGACAGAGACCCT
 S  A  K  Q  R  E  R  R  Y  S  T  V  F  L  A  L  D  R  D  P

         370        380        390        400        410        420
GCTGAGTCTATAGGGGGACCTGGAGGAACGGGGGGTGGTGGCAGTGGTGGGAGCAAGCGA
 A  E  S  I  G  G  P  G  G  T  G  G  G  G  S  G  G  S  K  R

         430        440        450        460        470        480
GAAGATAAGAAGTTGCAAAATGCCATTGTCAATGGGGTGCTCCAGAACACAGAGACCACC
 E  D  K  K  L  Q  N  A  I  V  N  G  V  L  Q  N  T  E  T  T

         490        500        510        520        530        540
AGTAAGGAGACAGAACCAGATTGCTTAGAGGTTAAAGAGCTGGCTCCACATCCATCTGGG
 S  K  E  T  E  P  D  C  L  E  V  K  E  L  A  P  H  P  S  G

         550        560        570        580        590        600
CTGCGCACAGCAGCAAGGTCCAAGCTTCGAAGACAGGAAGGTATTTCCCGCTTCTACATC
 L  R  T  A  A  R  S  K  L  R  R  Q  E  G  I  S  R  F  Y  I

         610        620        630        640        650        660
ATCCAAGTGGTGTTTCGAAATGCTCTGGAGATTGGGTTTCTGGTGGGCCAGTACTTTCTA
 I  Q  V  V  F  R  N  A  L  E  I  G  F  L  V  G  Q  Y  F  L

         670        680        690        700        710        720
TATGGCTTCAGTGTCCCAGGGTTGTATGAGTGCAACCGCTACCCCTGCATCAAGGAGGTA
 Y  G  F  S  V  P  G  L  Y  E  C  N  R  Y  P  C  I  K  E  V

         730        740        750        760        770        780
GAATGTTATGTGTCTAGACCTACGGAGAAGACGGTCTTTCTGGTGTTCATGTTTGCTGTG
 E  C  Y  V  S  R  P  T  E  K  T  V  F  L  V  F  M  F  A  V

         790        800        810        820        830        840
AGCGGCATTTGTGTGGTGCTCAATCTGGCTGAACTTAACCATCTGGGATGGCGGAAGATC
 S  G  I  C  V  V  L  N  L  A  E  L  N  H  L  G  W  R  K  I

         850        860        870        880        890        900
AAACTGGCTGTCCGGGGAGCCCAGGCCAAGAGGAAGTCAGTCTATGAGATTCGTAACAAA
 K  L  A  V  R  G  A  Q  A  K  R  K  S  V  Y  E  I  R  N  K

         910        920        930        940        950        960
GACCTGCCAAGAGTCAGTGTTCCCAATTTCGGCAGGACTCAGTCCAGTGACTCCGCCTAT
 D  L  P  R  V  S  V  P  N  F  G  R  T  Q  S  S  D  S  A  Y

   966
GTGTGAaagggcaggtttggggaaggcctgctaggtggcaagtagccccaaggcagagga
 V  *

tgactcgagggtgtacaggtttgccctcatttgcgtagtgctcgctgtgaaggtctagga
tacagatattaagaggtcgtcatcaacacaaaggagaggtagaactgtcatcacatgtca
caccatcagttctactgagaccaccgggtagagtgacaagatggctaagttggatgtcct
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tatttccaatttttttaaaactcaaattgcactggtgctgtttattaggggtgggggatgg
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tatgagctttgagatatttctgccttctagattatggcccg
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Fig. 1. cDNA and deduced amino acid sequences of rat Cx36. The rat Cx36 cDNA sequence is 96% homologous to the mouse 129 Sv Cx36 gene. The splice junction of exon 1 to exon 2, as deduced from analysis of the mouse Cx36 gene, is indicated by bold letters. Potential transmembrane regions, evaluated according to [20], are underlined. Each of the two putative extracellular domains contains cysteine motifs characteristic of other connexin sequences (cf. [1]). The nucleotide sequence is available from Gen-Bank/EMBL/DDBJ under accession no. Y16898. Potential binding sites for casein kinase I (S/E-X-X-S), casein kinase II (S-X-X-E), cAMP dependent protein kinase (R-X-S) and calmodulin dependent protein kinase (R-X-X-S) are indicated by boxes.

3. Results and discussion

We used degenerated primers derived from the skate Cx35 sequence [14] to amplify a cDNA, homologous to skate Cx35, by RT-PCR from mouse retina total RNA. We cloned an 802-bp fragment with 88% nucleotide sequence identity to skate Cx35 between primer USP2 and primer DSP3 (Cx35 USP2 was derived from DNA coding for the presumed first extracellular loop and Cx35 DSP3 from DNA coding for the end of the carboxy terminal region). This DNA fragment was used to screen libraries of rat brain cDNA and mouse genomic DNA. In addition it was used as probe for Southern and Northern blot hybridization.

We screened a λgt11 cDNA library isolated from rat brain RNA at day 18 p.c. with the mouse 802-bp PCR fragment. A specifically hybridizing cDNA clone was isolated and sequenced on both strands (Fig. 1). The central of three ATG codons was found to be located after a consensus initiation region of translation [18], followed by an open reading frame coding for a protein of 321 amino acids with a predicted molecular mass of 36 045 kDa. We designated this new connexin as rat Cx36 according to the nomenclature suggested by Beyer et al. [19].

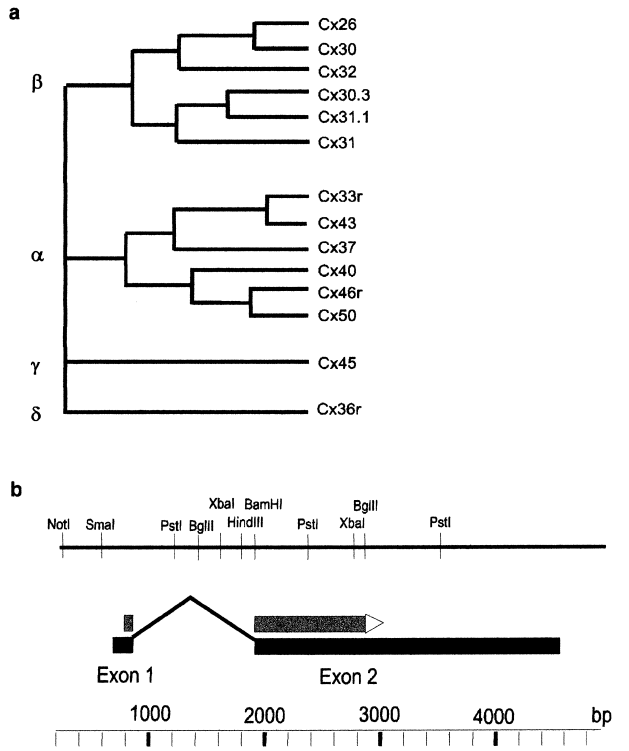

Fig. 2. a: Phylogenetic tree of connexins. The dendrogram was deduced by CLUSTRAL analysis of total mouse protein sequences, except Cx33, Cx36 and Cx46 for which rat (r) sequences were used, since the corresponding mouse sequences have not been published. The unique features of the murine Cx36 gene suggest to place it into a new δ subgroup of the connexin gene family, since Goodenough et al. [1] have already listed the sequence of Cx45 separately from the α and β subgroups of connexin genes. b: Genomic map of the mouse Cx36 gene. The positions of exon 1 and exon 2 are indicated by solid boxes. The coding region in exons 1 and 2, shown in the upper part of this figure, is indicated by hatched boxes. Additionally, a restriction map is shown based on results of Southern blot hybridization and sequence analysis. The coding region of the murine Cx36 gene is interrupted by a 1.14-kb intron.

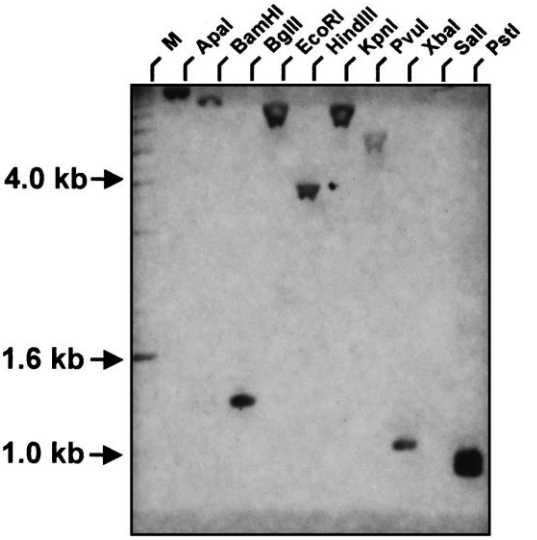

Fig. 3. Southern blot hybridization of mouse 129 Sv DNA extracted from embryonic stem cells. Genomic DNA was digested with the listed restriction endonucleases, electrophoresed and subjected to Southern blot hybridization using the 802-bp mouse Cx36 cDNA fragment as a probe. The pattern of single bands on the autoradiograph suggests that only one copy of the Cx36 gene exists in the mouse genome. The *Pst*I 0.9- and 0.8-kb double fragment is due to a *Pst*I restriction site in exon 2.

Together with 120 bp cloned 5′-UTR, 0.82 kb cloned 3′-UTR and 0.96 kb of the coding region, the identified rat Cx36 cDNA sequence is about 1.1 kb shorter than the 2.9-kb mouse Cx36 mRNA (Fig. 1), suggesting that the untranslated regions are not yet completely known. The analysis of the deduced rat Cx36 amino acid sequence shows the typical features of a connexin protein: it contains four putative transmembrane regions and two putative extracellular loops with conserved cysteine residues, in analogy to other connexins [7]. The putative transmembrane domains (underlined in Fig. 1) were predicted by the algorithm of Rao and Agros [20].

Rat Cx36 shows similar levels of amino acid identity compared to β group connexins, i.e. Cx26 (65.9%) or Cx32 (64.7%), and to α group connexins, i.e. Cx46 (67.0%) and Cx50 (67.7%) [21] (Fig. 2a). In comparison to α and β connexins, both Cx36 and Cx45 have additional sequence information within their putative cytoplasmatic loop, so that the unusually long putative cytoplasmic loop of 79 amino acid residues in Cx45 is further extended to 99 amino acid residues in Cx36. Of all other connexins, however, only the putative cytoplasmatic C-terminal region of Cx26 is shorter than that of Cx36. In this short sequence of Cx36, potential recognition sites [22] for casein kinases I and II, cAMP dependent protein kinase, and calmodulin dependent protein kinase II were found, whereas no consensus sequences for protein kinase C or *N*-glycosylation were detected (see Fig. 1). Another special feature of the Cx36 protein is the stretch of 10 glycines within 13 amino acid residues between positions 125 and 137 of the putative cytoplasmatic loop. Thus we suggest that Cx36 forms a new δ subdivision of the connexin gene family, in addition to the α, β and γ subgroups previously defined [1].

After screening a genomic library from the 129 Sv mouse strain with the 802-bp Cx36 DNA probe we obtained and partially sequenced a 10.5-kb DNA fragment. In the coding region we noticed two differences to the mouse genomic se-

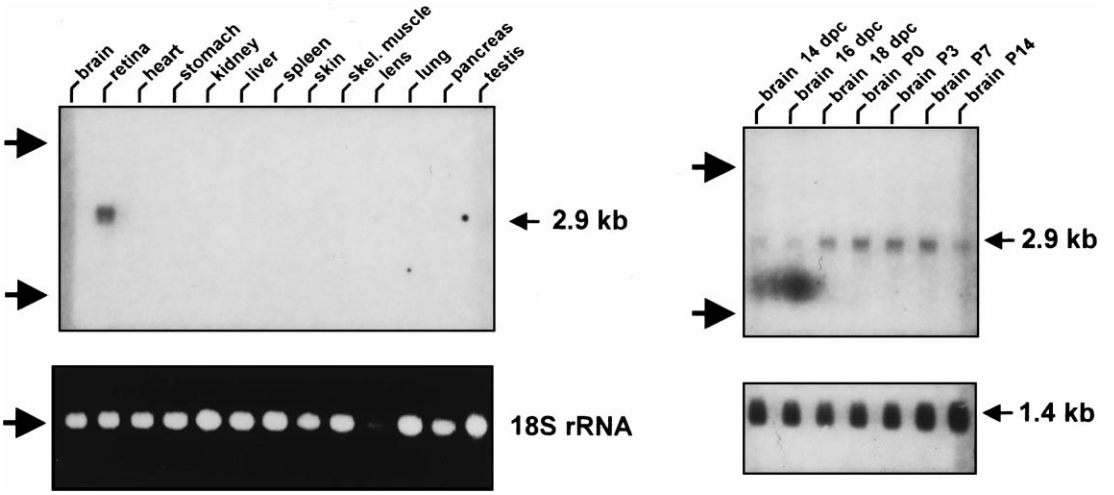

Fig. 4. Northern blot hybridization of total RNA from 13 adult mouse tissues and seven stages of mouse brain development. A 2.9-kb band was detected after 1 week of exposure only in RNA of adult retina (left) and of all developmental stages of mouse brain tested (right). The right blot was standardized by rehybridization to a DNA probe coding for human glyceraldehydephosphate dehydrogenase (GAPDH) [17]. A densitometric evaluation indicated that the Cx36 mRNA signal showed the following relative intensities during brain development: 14 dpc 20%; 16 dpc 24%; 18 dpc 58%; P0 81%; P3 93%; P7 100%; P14 40%. After two weeks of exposure, a faint 2.9-kb band was detected in RNA of adult brain with an intensity of about 8% (not shown). The lower part left illustrates the amounts of 18S rRNA in the ethidium bromide stained agarose gel indicating that, with the exception of lens, about equal amounts of total RNA were electrophoresed.

quence published by Condorelli et al. [15] which led to the amino acid alterations $Val^{76}Ile$ and $Met^{149}Ile$. We noticed that the deduced mouse Cx36 coding region was interrupted by an 1.14-kb intron, which separated the first 71 bp, starting with ATG, from the rest of the coding region. This genomic structure of the murine Cx36 gene (Fig. 2b) is similar to the genomic organization of the skate Cx35 gene [14] and differs from other murine connexin genes (cf. [23]). Consensus splice donor and acceptor sites are located at the borders of the intron. In addition, a possible lariat consensus motif [24] was recognized near the 3′ end of the intron (not shown). The putative splice junctions in the rat cDNA are marked with bold letters (Fig. 1). We found one amino acid difference (Ala→Thr) at position 175 between rat and mouse Cx36, which show 96% nucleotide sequence identity. The genomic organization of the mouse Cx36 gene (cf. Figs. 1 and 2b) shows that the first exon appears to code for the putative N-terminal domain which has been shown with other connexins to mediate different voltage gating properties [25].

Mouse genomic DNA was digested with several restriction endonucleases. Southern blot hybridization indicated that a single copy of the Cx36 gene exists in the mouse genome (Fig. 3). Under these conditions single DNA fragments of 1.4, 6, 4 and 1.1 kb were detected after digestion with *Bgl*II, *Eco*RI, *Hin*dIII and *Xba*I, respectively. Only *Pst*I digestion yielded two fragments of 0.9 kb and 0.8 kb which indicates a *Pst*I cleavage site in the coding region.

We analyzed the transcriptional expression of the new connexin gene in 13 adult mouse tissues and at seven stages of mouse brain development (Fig. 4). Only one signal of 2.9 kb was found in adult retina but not in 11 other adult mouse tissues which is in accordance with the retina specific expression of skate Cx35 [14], although these authors detected two transcripts of 1.95 and 4.6 kb. After two weeks of exposure, however, we found a weak signal in adult mouse brain RNA at 2.9 kb (result not illustrated).

During mouse brain development, transcriptional expression of the new connexin gene was up and down regulated. After standardization with a GAPDH probe, the densitometric analysis of Cx36 mRNA expression revealed a gradual increase beginning with 20% at day 14 p.c. and reaching 100% expression at postnatal day P7. Subsequently, the expression level declined to 40% and reached the basal value of about 8% in adult brain. Highest expression of Cx36 mRNA was found in adult mouse retina, where it was 1.5-fold more abundant than in postnatal mouse brain. Interestingly, Rörig et al. [26] have observed decreasing transfer of microinjected neurobiotin among rat neurons in slices of the developing neocortex between postnatal day 7 and 15. Possibly this effect could be due to declining expression of Cx36.

Very recently, Condorelli et al. [15] have also cloned Cx36 from the mouse genome and have shown by in situ hybridization that Cx36 is expressed in adult rat brain neurons, especially in the inferior olive, olfactory bulb and in CA3/CA4 hippocampal regions. Cx36 appears to be involved in neuronal coupling not only in specialized adult neurons, but also seems to play an important role during brain development. The regulatory mechanisms that direct expression of Cx36 in different regions of the central nervous system and at different stages of neural development remain to be elucidated.

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