ELSEVIER

Gene 251 (2000) 123–130

GENE
AN INTERNATIONAL JOURNAL ON GENES, GENOMES AND EVOLUTION

www.elsevier.com/locate/gene

Genomic organization and chromosomal localization of the mouse *Connexin36* (*mCx36*) gene

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Received 14 February 2000; received in revised form 10 April 2000; accepted 28 April 2000
Received by E. Boncinelli

Abstract

Connexin36 (*Cx36*) is a new connexin that was recently cloned in mouse, rat and human. It is highly expressed in neurons of the CNS. To gain insight into the transcriptional regulation of this gene, we have cloned the genomic region containing the entire *mCx36* gene and sequenced about 7.6 kb around the coding region. The computer analysis of this sequence was helpful in defining putative regulative sequences. Using both 5′-RACE and RNAse protection assay, we have mapped the transcription starting site commonly used in both adult olfactory bulb and brain, in position −479 from the ATG. By 3′-RACE, we defined the polyadenylation site used that is located 1436 nt downstream the stop codon. The expected transcript is 2875 nt long and is consistent with the 2.9 kb transcript found in the same tissues by Northern blot. Finally, we have mapped *mCx36* on chromosome 2 in the position F3 in a region that is synthenic to human chromosome 15q14, where the human *Cx36* gene has been recently mapped.

Keywords: FISH; Gap junction; Mouse chromosome 2; Untranslated regions

1. Introduction

Gap junctions form an aqueous path that permits the passage of ions, small metabolites and second messengers between the cytoplasm of adjacent cells. They have a major role in synchronizing activity between cells through metabolic co-operation and long-range signalling (Saez et al., 1989; Carter et al., 1996; Goldberg et al., 1998; Kam et al., 1998).

Gap junctions result from the association of two half channels, named connexons, contributed separately by each of the two participating cells. Each connexon, in turn, is a multimeric assembly of structural proteins (six) called connexins (Cx) that form a multigene family, whose members are distinguished according to their predicted molecular mass in kDa.

Twenty different connexins have already been cloned so far in mammals (White and Paul, 1999). They are characterized by four transmembrane domains needed for membrane anchoring, spaced by two extracellular domains used for interaction with connexins in the counterpart cell. Between transmembrane domains two and three, connexins present a specific cytoplasmatic loop, suggested to be important for pH gating of the channel (see Bruzzone et al., 1996).

Gap junctions are widely distributed in various organs and tissues, and it has been suggested that all cell types of mammals express intercellular channels (Bruzzone et al., 1996). Only recently, a new connexin specific for neuronal cells, named *Cx36*, has been cloned both in mouse and rat (Condorelli et al., 1998; Sohl et al., 1998). No insight into the untranslated (UTR) regions of the rat or mouse *Cx36* gene and no specific analysis of the transcription starting site and the promoter sequences have been given in previous studies.

The present study focuses on the cloning of the genomic region containing the entire *mCx36* gene, the analysis of its genomic organization, and the chromosomal mapping in the mouse.

Abbreviations: B, adult brain; Cx, connexin; L, liver; OB, adult olfactory bulb; P, probe.

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0378-1119/00/$ - see front matter
PII: S0378-1119(00)00202-X

2. Materials and methods

2.1. Isolation of genomic clones

Genomic clones were isolated from a mouse 129/Sv genomic phage library. The probe used in the screening was a 302 bp PCR fragment encoding the intracellular loop of the *mCx36* sequence (position 283–585 in the mouse sequence), subcloned in pBluescript KS^+ vector (Stratagene, San Diego, CA). Positive clones were first purified by several rounds of screening, then characterized by restriction enzyme digestions and Southern blotting. Suitable fragments were subcloned into pBluescript SK vector and sequenced double stranded using the ThermoSequenase kit (Amersham).

2.2. RNA isolation from mouse tissue

Total RNA was isolated, as described by Chomczynski and Sacchi (1987), from adult male mouse olfactory bulb (OB), total brain (B), and liver (L).

2.3. Northern blot analysis

Northern blot analysis of *Cx36* mRNA was performed as previously described (Condorelli et al., 1998), using the same probe used for library screening (see Section 2.1). *Cx36* and β-actin probes were ^{32}P-labelled (specific activity: 10^9 cpm/mg DNA) by random priming (Gibco-Life Technologies).

2.4. 5′- and 3′-RACE

The 5′-end of mouse *Cx36* was isolated by 5′-RACE (Rapid amplification of cDNA ends, Gibco/BRL) following instructions supplied by the manufacturer. Briefly, cDNA was synthesized by Superscript II™ reverse transcriptase using 1 μg of total RNA and 1 μM antisense primer Rt01 (5′-AATGGCCACAATGAGTAT-3′). The RNA template was removed by RNAse H, and the resulting cDNA product was purified on a GlassMAX cartridge. The cDNA was tailed with dCTP using terminal transferase. For each tailed cDNA, a control sample to which no terminal transferase was added, was included and used as negative control in subsequent PCR reaction. Both tailed cDNA templates and their untailed controls were subjected to two consecutive rounds of PCR. In the first round of PCR, the Anchor Primer provided with the 5′-RACE system (Gibco/BRL), which consisted mainly of dGTP residues to allow annealing with the dCTP tail, was used together with the Cx36 specific antisense primer Rt02 derived from the coding sequence (5′-TCTCCAAGATGGTCCATT-3′). The second round of PCR was performed with the UAP (Universal Amplification Primer), also provided with the 5′-RACE system, and designed to anneal to restriction sites incorporated into the PCR product by the Anchor Primer, together with antisense primer Rt03 (5′-GGCCCTCCCCCTTTATTGCACTTA-3′), situated 150 bp upstream of the ATG.

The 3′-end of mouse *Cx36* was isolated by 3′RACE (Rapid amplification of DNA ends, Gibco/BRL) following the instructions supplied by the manufacturer. Briefly, cDNA was synthesized by Superscript™ reverse transcriptase using 1 μg of total RNA and the oligo-dT antisense primer provided with the kit. After the first-strand cDNA synthesis, the RNA template was removed by RNAse H. Both cDNA templates and their controls were subjected to a nested round of PCR, using sequentially one mCx36 specific sense primer (F04: 5′-GCTGTGAGCGGCATTTGTGTGG-3′) or (F05: 5′-GAGAGGTAGAACTGTCATCACG-3') and an adaptor primer for the poly(A) tail, provided by the kit manufacturer. PCR products were separated on 1% agarose gel, purified using Jetsorb (Genomed) and cloned into TA cloning (Invitrogen). For each PCR product, multiple clones were sequenced using Thermosequenase polymerase (Amersham), according to the protocol supplied by the manufacturer.

The positions for all primers used are indicated in Fig. 3B.

2.5. RNAse protection analysis

For RNAse protection, we used a fragment *Not*I–*Sma*I of 536 bp (Fig. 3C) located 71 bp upstream from the ATG start codon, as a probe. RNase protection was performed as follows. Antisense RNA probes with a high specific activity (8×10^8 cpm/μg) were synthesized using the Riboprobe Gemini System II (Promega). Approximately 2×10^4 cpm of the specific radiolabeled riboprobe and total RNA (40 μg) were added together, denaturated at 85°C for 5 min and hybridized overnight at 52°C in 80% formamide, 40 mM PIPES, pH 6.4, 1 mM EDTA, pH 8.0, 0.4 M NaCl. At the end of the hybridization, the samples were treated with 3 μg/ml of RNAse T_1 and 30 μg/ml of RNAse A in 300 mM NaCl, 10 mM, Tris pH 7.4, 5 mM EDTA, pH 7.5, for 2 h at 32°C. The samples were then incubated in 1 μg/ml of proteinase K, 0.2% SDS for 1 h at 37°C, extracted with phenol–chloroform–isoamilic, precipitated with ethanol and resuspended in formamide dye (90% formamide, 1 mg/ml of xilene cyanol FF, 1 mg/ml of Bromophenol Blue, 10 mM Tris pH 7.5). Samples were finally denatured, and protected fragments were analysed on a 6% gel.

2.6. Genomic localization

FISH experiments were performed with the entire phage 3 (Fig. 1) containing an 15.5 kb genomic insert spanning the whole coding region and 10.5 kb upstream

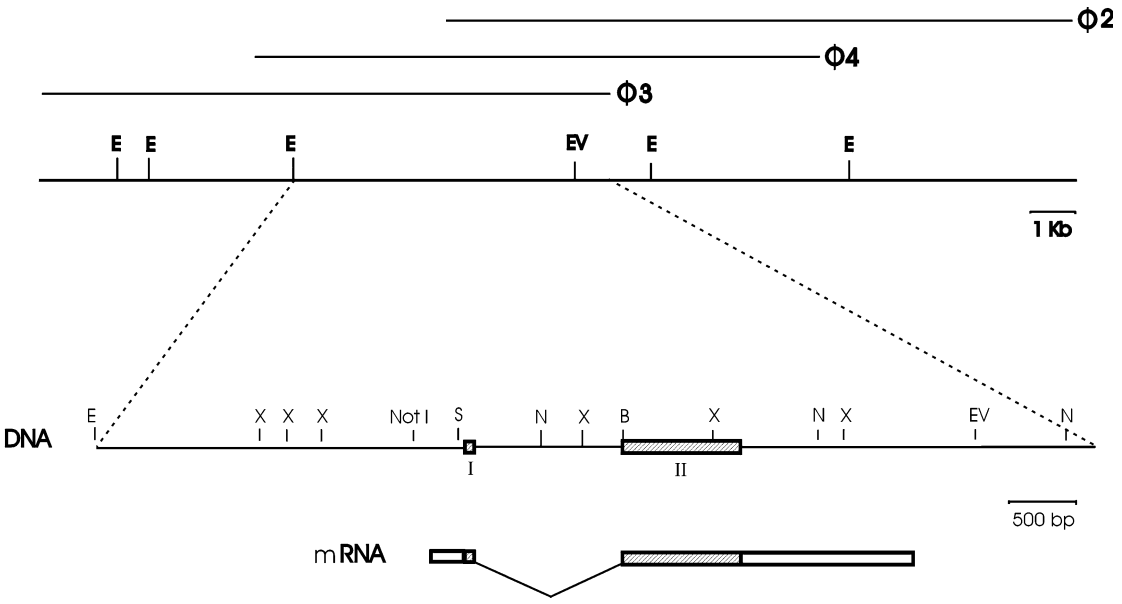

Fig. 1. Organization of the genomic region containing Cx36 in the mouse. At the top, genomic inserts from three isolated phages are shown by horizontal lines. The genomic region with identified restriction enzymes sites is indicated in the center. The sequenced region is enlarged at the bottom of the figure, under which the putative mRNA product is figured. Hatched boxes indicate coding regions, and empty boxes represent 5′- and 3′-UTR, as found by-RACE and RNAse protection. E = *Eco*RI, EV = *Eco*RV, B = *Bam*HI, S = *Sma*I, N = *Nde*I, X = *Xba*I.

of the ATG and 2.5 kb downstream the TGA. This probe (200 ng/slide) was labeled by nick translation with biotin-16-dUTP (Boehringer Mannheim) according to the manufacturer's protocol. Hybridization was performed on fibroblasts metaphases from C57BL/6J male mice at 37°C in 50% formamide/2× SSC with 500× salmon sperm DNA and followed by post-hybridization washes at 37°C in 50% formamide/2× SSC (3×5 min) and, subsequently, in 2× SSC (3×5 min). The detection was performed using the fluorescein avidin DCS (Vector) with three amplification steps. Chromosomes were counterstained with propidium iodide (1 μg/ml), banded with diaminophenylnindole (DAPI), and mounted in antifade solution (Vectashield mounting medium, Vector). The screening of slides for gene assignment was performed as described previously (Banfi et al., 1996). Briefly, only those chromosomes with signals present on both chromatids at the same band position were taken into consideration. Among 50 suitable metaphases, 21 signals were assigned at 2F3 (42%) (see Fig. 4). No clusters of signals were detected on other chromosomes.

3. Results and discussion

3.1. Genomic organization of the mCx36 gene

To isolate the mouse *Cx36* gene, a mouse 129/Sv genomic DNA phage library was screened, using a ^{32}P-labeled mouse *Cx36* fragment specific for the intracellular loop as a probe (see Section 2.1). Under high-stringency hybridization and washing conditions, a total of three positive clones were isolated, from approximately 8×10^6 independent clones. Restriction mapping and Southern blot analysis revealed that the three phage clones were overlapping and spanning about 28 kb of contiguous genomic DNA as shown in Fig. 1.

Further characterization by subcloning and sequencing the restriction fragments positive by Southern blotting to the *Cx36* cDNA demonstrated that the overlapping genomic inserts of the phagic clones cover the entire genomic region containing the *mCx36* gene.

We have sequenced, in double strand, the region containing the *mCx36* gene, within a *Eco*RI–*Eco*RV genomic fragment (Fig. 1) and aside regions, for a total of 7.640 bp (GenBank Accession No. AF226992) (Fig. 2A and B). The sequences of exon/intron boundaries were determined by comparing the nucleotide sequences of the *Cx36* gene with those of the Cx36 cDNA (Condorelli et al., 1998). The sequences for all splice sites are well conserved and conformed to the GT/AG rule (Breathnach and Chambon, 1981). The translation initiation and termination codons (ATG and TGA) are located in exon 1 and 2, respectively (Fig. 1). Several base differences have been found within the sequence when compared to the previously published sequence (GenBank Accession No. AF016190).

Usually, connexins contain all the coding sequence within a single exon and have the 5′-UTR region in a single upstream exon that can be alternatively spliced using different promoters, as is the case for *Cx32*, which are conserved between different species (Neuhaus et al., 1995, 1996; Duga et al., 1999). *Cx36* is peculiar in its genomic organization by the fact that its coding region

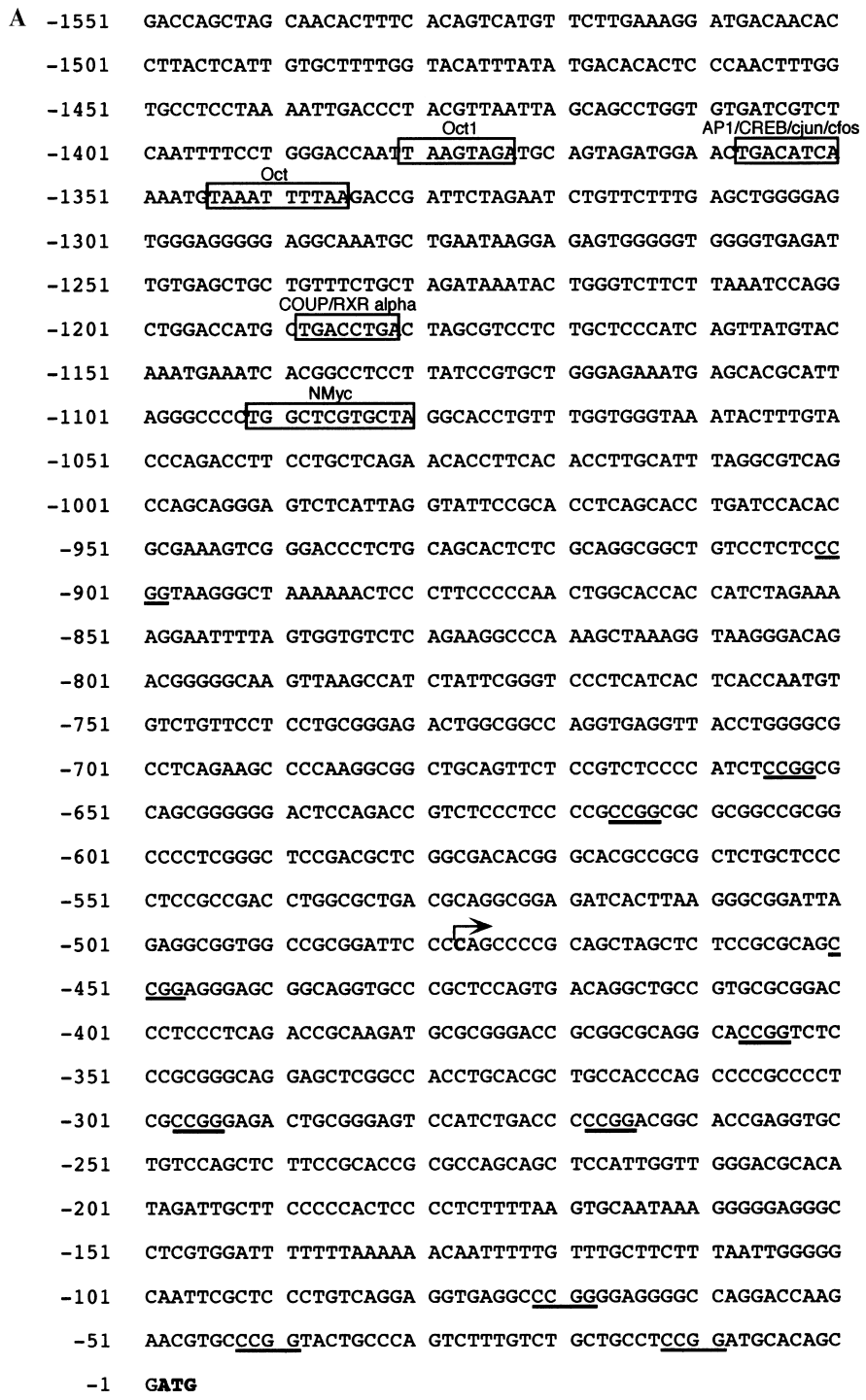

Fig. 2. Annotated partial sequence of a *Eco*RI–*Eco*RV genomic fragment containing the mouse *Cx36* gene. The complete sequence has been deposited in GenBank (Accession No. AF226992). (**A**) DNA sequence located 5′ upstream the ATG translation start site. The transcription starting site found by 5′-RACE and RNAse protection is shown in bold with an arrow. Putative significant binding sites within the 5′ region around the transcription start site are indicated. CG sequences are underlined. (**B**) DNA sequence located 3′ downstream of the TGA stop codon. The polyadenylation signal found by 3′-RACE is indicated in a square. The polyadenylation site is indicated by an arrow. The GT sequence downstream of the polyadenylation site is underlined. Alternative polyadenylation signals are also indicated by dotted line squares.

is not contained in a unique exon but is spliced by a short intron of 1140 bp in mouse (Fig. 1) and 1048 bp in human (Belluardo et al., 1999; our data). Comparison between coding regions of already identified Cx36 homologs in different species have indicated that there are highly conserved regions.

For human *Cx43*, a processed pseudogene has also been found (Fishman et al., 1991). No pseudogenes

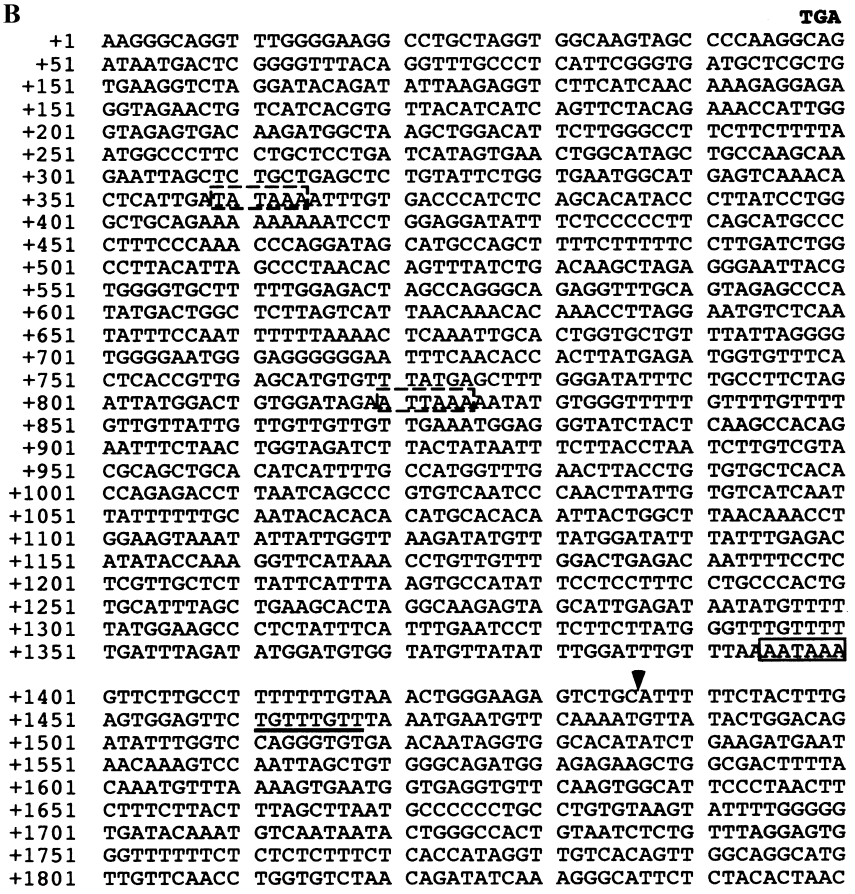

Fig. 2. (*continued*).

have been found by genomic Southern blotting using the coding sequence of *Cx36* as a probe (data not shown).

3.2. Mapping of the transcription starting site and polyadenylation site

Previous studies of *Cx36* both in rat (Condorelli et al., 1998), mouse (Condorelli et al., 1998; Sohl et al., 1998) and human (Belluardo et al., 1999) have not given any insight into the UTR regions of the *Cx36* gene, and no specific analysis of the transcription starting site was performed.

To define the transcription starting sites and to determine whether there are any other exons in addition to those already identified, we performed both 5′- and 3′-RACE on total RNA from different brain regions and checked the results by RNAse protection.

The putative 5′-UTR region was amplified by the PCR method 5′-RACE, using RNA from both total brain and adult olfactory bulb as templates, since these tissues had been shown by Northern blot analysis to express *Cx36* mRNA (Condorelli et al., 1998) at high level (Fig. 3A).

We have used a nested strategy, with the reverse most 5′ oligonucleotide located just upstream of the ATG start codon. All the primers used are indicated in Fig. 3B. A PCR product migrating between DNA marker fragments of about 300 bp of an agarose gel (Fig. 3B) was detected after two rounds of PCR with nested primers (Rt03 and Rt02 in Fig. 3B), and no product was detected in the negative control PCR reaction. The PCR product was cloned, and the cDNA sequence upstream of the translation start codon ATG was found to be colinear to the genomic sequence shown in Fig. 2A. The putative transcription start site was positioned at the C residue in position −479 (Fig. 2A).

To check whether the 5′ identified by 5′-RACE corresponds to a putative transcription site for mCx36, we used a specific probe (see Fig. 3C and Section 2.5), spanning the 5′ region in an RNAse protection assays on different total RNA from isolated adult CNS regions (olfactory bulb and brain).

RNAse protection assay showed a single band of 408 nt long (Fig. 3C). We therefore conclude that *mCx36* mRNA transcription starts at the C-residue (−479), indicated in Fig. 2A, although possible minor transcription start sites cannot be completely ruled out.

The putative 3′-UTR was amplified by the PCR method, 3′-RACE, from total brain RNA. The cDNA

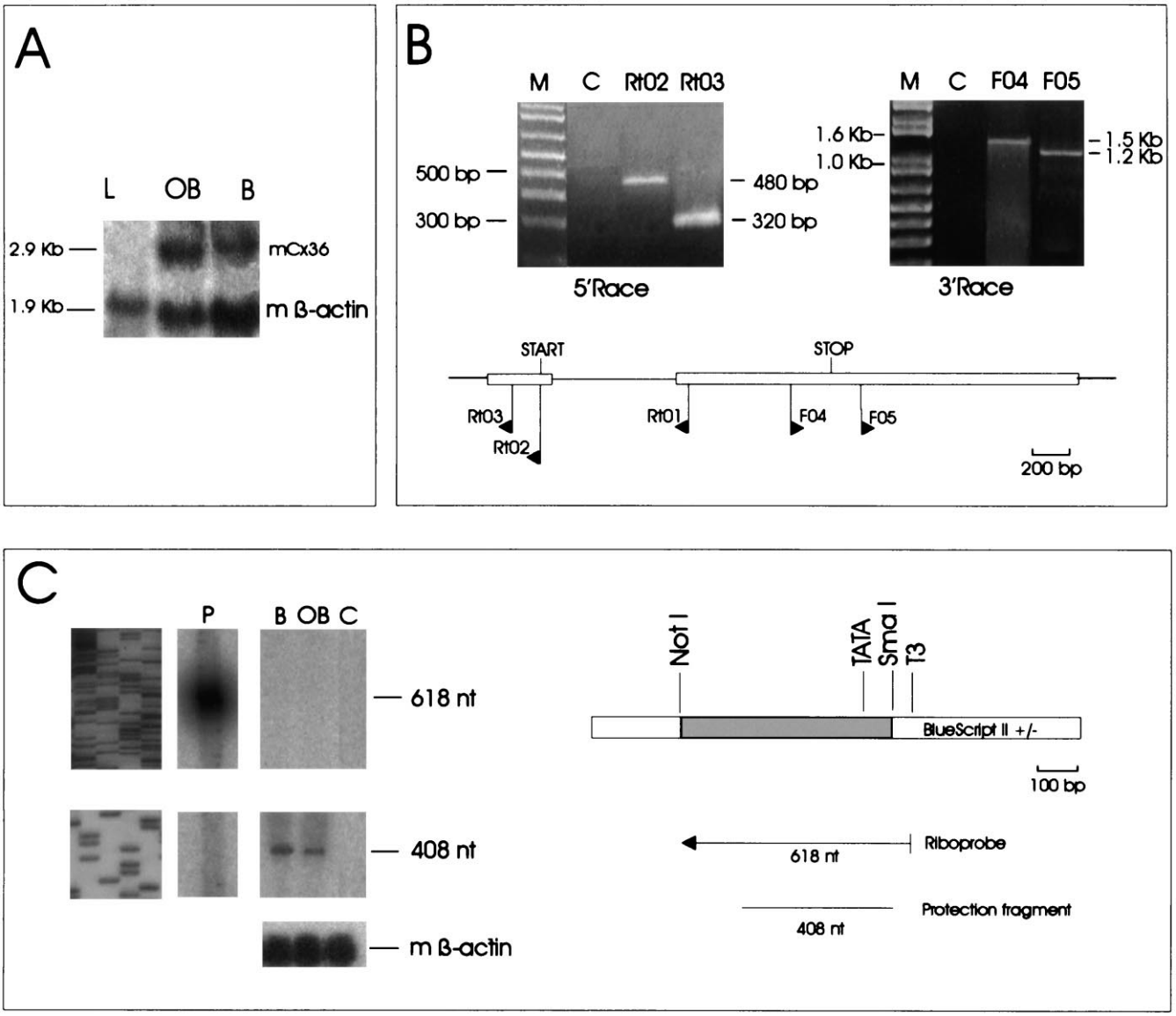

Fig. 3. (**A**) Northern blot A single band at 2.9 kb is detected by Northern blot, corresponding to *mCx36* transcript in adult olfactory bulb (OB), adult brain (B). No hybridization signal was found in the adult liver (L). β-actin probe was used for normalization. (**B**) 5′ and 3′-RACE. Specific bands obtained by 5′ and 3′-RACE are indicated, respectively, on the left- and right-hand side of the figure. Specific oligonucleotides used are indicated in the map drawn (see Sections 2.4 and 3.2). The negative controls are indicated as C. (**C**) RNAse protection. Thirty micrograms of total RNA from adult brain (B), olfactory bulb (O) and yeast RNA as a control (C) were hybridized with a ^{32}P labeled riboprobe spanning nt −607 to −71 upstream of the ATG of the *mCx36* gene, and treated with a mixture of RNAse A/T1. A single protected band of 408 nt has been found both in the adult brain (B) and in the olfactory bulb (OB). (P) denotes the probe. The diagram on the right shows the position of the probe and the protected fragments on the *mCx36* genomic region

template and their controls were subjected to a double nested round of PCR using both a *mCx36* specific sense primer (F04 and F05; see Fig. 3B) and an adapter primer for the poly(A) tail region. The cloned 3′-RACE product appeared to be, respectively, 1.5 kb (primer F04) and 1.2 kb long (primer F05) (Fig. 3B). Its sequence was compared to the genomic sequence and was shown to be co-linear to it, indicating that for the *mCx36* gene, polyadenylation occurs at the dinucleotide (CA) located at position 1436 nt downstream of the stop codon. The CA dinucleotide is preceded by the polyadenylation signal AATAAA and followed by a GT-rich sequence (tgtttgtt), as indicated in Fig. 2B. The two exons are therefore, respectively, 550 and 2325 bp long.

3.3. Analysis of 5′- and 3′-UTR and regulatory sequences

Northern blot analysis in different mouse tissues indicates that mCx36 is present in a single transcript, estimated to be 2.9 kb in length (Sohl et al., 1998; and Fig. 3A). Both the 5′-RACE and the RNAse protection

assay on RNA from adult olfactory bulb and brain indicate that the transcription starting site is located −479 from the ATG and that the polyadenylation site used is at position +1436 downstream of the stop codon. The expected transcript is then of 2875 nt, which matches the 2.9 kb transcript length indicated by Northern blot (Fig. 3A). A computer analysis (see Section 3.4) of the genomic sequence upstream of the start codon has identified at least two other TATA boxes and two polyadenylation sites, as indicated in Fig 2A and B, respectively. It is possible that these other sites are used in few transcripts, that we were not able to detect, due to the sensitivity of the assays used, or are used in tissues not analyzed in this study. We have also found that 5′ upstream to the translation site, there is a region that is rich in binding sites for transcription factors, opening up the possibility that this region is part of the promoter region of the gene. These data indicate that *Cx36* is different, both in genomic organization and in transcription regulation, from previously identified connexins. To understand whether the exon containing the ATG could have been alternatively spliced in some tissues, we have specifically used the first primer within the second exon-coding region, so as to retrotranscribe all the population of mRNA containing *Cx36* second exon sequences (that characterize the *Cx36* gene itself) (Fig. 3B). A round of 5′-RACE using a second reverse primer within the same second exon (data not shown), has not yielded any product containing an alternative 5′ exon, indicating that in the tissues under analysis (adult total brain and olfactory bulb), there is no alternative splicing.

3.4. CpG islands, and binding sites

We have analyzed the genomic region upstream of the ATG and around the transcription start site using several computer programs such as TESS (at http://www.cbll.unipenn.edu) and MatInspector (at http://genomatix.gsf.de; Quandt et al., 1995).

The 5′ flanking region of the mouse *Cx36* gene is extremely GC-rich (75% in the 250 bp just upstream the ATG start codon), and there are 10 *Hpa*II/*Msp*I sites in the 800 bp upstream of the ATG, indicating that it could be a CpG island (Fig 2A). CpG islands are clusters of unmethylated CGs that are located around the transcription start site of many genes, including tissue-specific genes. We have localized the transcription start site for mCx36 and within this region.

By sequence analysis, we found several potential binding sites for transcription factors, including AP-1, AP-2, TFIID and Sp1. Of particular interest, there are three motifs among others. One is a COUP and RXR alpha motif (TGACCTGA) in position −1190/−1183 from the ATG (see Fig. 2A). Other connexins, such as Cx43, are known to respond, at least in vitro, to retinoic signalling. The other motif is an Oct1 binding site (TAAGTAGA: position −1382/−1375 from the ATG) and a general Oct binding site (TAAATTTTAA in position −1346/−1337 from the ATG). Moreover, a AP-1/CREB/c-fos/c-jun motif (TGACATCA) has been found in position −1359/−1352 from the ATG, and an Nmyc site (tggctcgtgcta) in position −1093/−1080. Due to the fact that the 5′-UTR regions of both rat and human *Cx36* have yet not been published, we were unable to perform any analysis of the evolutionary conservation of these sequences.

3.5. mCx36 is localized on Chr 2 in the mouse

We used FISH (Fig. 4) hybridization on mouse chromosome to localize *mCx36* on chromosome 2 in the portion F3, in a region that is synthenic to human chromosome 15q14–15 (NCBI GenBank, Mouse to human Homology Map at http://www.ncbi.nlm.nih.gov and MGD at http://www.informatics.jax.org). Recently,

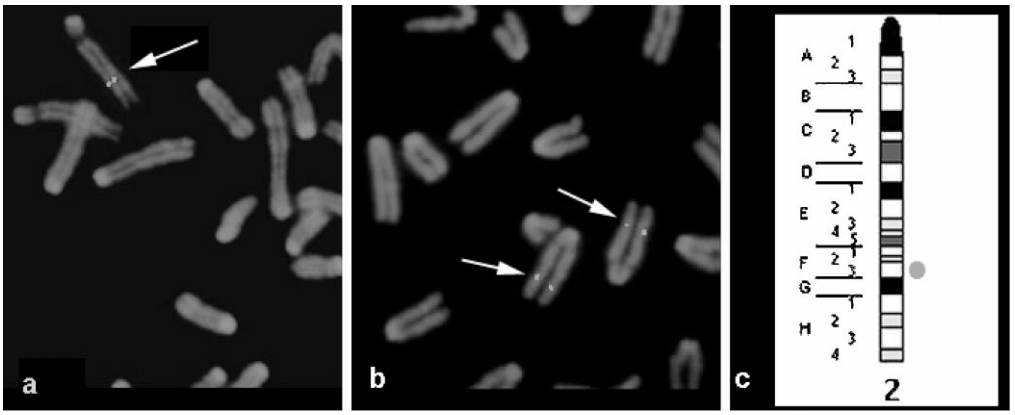

Fig. 4. FISH. (**a**, **b**) White arrows indicate the signal on chromosome 2 in two partial metaphases. (**c**) Chromosome 2 ideogram with the signal localized at 2F3.

the human homolog of *Cx36* has been cloned (Belluardo et al., 1999 and our manuscript, in preparation) and found to map on chromosome 15q14. Specifically, mCx36 results localized in a region between SGNEI in position 2E5 (cM64) and CKMT1, EPB42, RECA, TYRO3 all in 2F (cM67), possibly near RYR3. The human homologs of these genes have been mapped on human chromosome 15 between q13 and q15, indicating that this region is highly conserved during evolution in its genomic organization.

4. Conclusions

A new connexin specific for neuronal cells, named *connexin36* (*Cx36*), was recently cloned in rat, mouse and humans. No insights in the UTR regions and no specific analysis of the transcription starting site and promoter sequences were given in these studies or the chromosomal location of the *Cx36* gene in the murine genome. In the present study, we have addressed these points. We cloned a mouse genomic region spanning about 28 kb around the *Cx36* gene, and we have sequenced several kilobases upstream and downstream of the coding region. We have mapped the mouse gene on mouse chromosome 2 in the region F3 in a position of homology with the human gene, recently mapped at position 15q14. The analysis of the flanking sequences has given insights into the regulatory regions of the *mCx36*, and we have found several putative binding sites for regulative proteins. Finally, we have identified the transcription starting site and the polyadenylation site used in the adult brain and olfactory bulb. These data would be on the basis of any functional analysis of the promoter region, both in vivo and in vitro, and will help in defining a strategy for the production of homologous recombinant transgenic mice.

Acknowledgements

We wish to thank Dr Antonio Simeone for providing us with the mouse Sv/129 genomic library, and Dr Dario Acampora for his invaluable help and suggestions during the initial part of this work. This work was supported by CNR Grant No. 98.02996.CT04, Italian Theleton Grant No. D77 and EU BIOTECH2 Grant No. BIO4-CT98-0309.

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