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Structure and Chromosomal Localization of the Human Homeobox Gene Prox 1

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The genomic organization and nucleotide sequence of the human homeobox gene Prox 1 as well as its chromosomal localization have been determined. This gene spans more than 40 kb, consists of at least 5 exons, and encodes an 83-kDa protein. It shows 89% identity with the chicken sequence at the nucleotide level in the coding region, while the human and chicken proteins are 94% identical. Among the embryonic tissues analyzed (lens, brain, lung, liver, and kidney), the human Prox 1 gene is most actively expressed in the developing lens, similar to the expression pattern of the chicken Prox 1 gene. The Prox 1 gene was mapped to human chromosome 1q32.2–q32.3.

INTRODUCTION

It is well known that homeodomain proteins play an essential role in the determination of cell fate and the establishment of body plan (see McGinnis and Krumlauf, 1992; Kenyon, 1994; Scott, 1994). Moreover, homologous homeobox genes are often involved in the development of analogous organs in evolutionarily distant organisms. For example, mammalian Pax-6 (Hill *et al.*, 1991; Ton *et al.*, 1991) and the homologous gene in *Drosophila, eyeless* (Quiring *et al.*, 1994), are absolutely essential for early eye development in spite of the tremendous differences in the eye organization of vertebrates and insects (see Halder *et al.*, 1995; Tomarev and Piatigorsky, 1996, for review). Very often, mutations in homeobox genes that are highly expressed in a particular organ can lead to developmental anomalies in this organ. For instance, mutations in the human Pax-6 gene can lead to aniridia (Ton *et al.*, 1991; see Hanson and Heyningen, 1995, for review), while some mutations in *eyeless* result in a characteristic reduction in the size of the compound eye, which, in the most extreme cases, may be missing completely (see Lindsley and Zimm, 1992).

Recently, we isolated and characterized a cDNA encoding the chicken homeodomain protein, Prox 1 (Tomarev *et al.*, 1996). This gene is highly expressed in the developing lens, retina, and pancreas of chickens, with lower levels detectable in the developing brain, heart, and skeletal muscles. In the mouse, only a portion of the Prox 1 protein sequence corresponding to the homeo- and C-terminal prospero domain was reported (Oliver *et al.*, 1993). Mouse Prox 1 expression was detected in the young neurons of the subventricular region of the CNS, as well as the developing lens and pancreas (Oliver *et al.*, 1993). In *Drosophila*, a likely homolog, *prospero,* is expressed in the developing CNS, lens-secreting cone cells of the eye, and midgut (Doe *et al.*, 1991; Vaessin *et al.*, 1991; Matsuzaki *et al.*, 1992; Oliver *et al.*, 1993). During *Drosophila* CNS development, *prospero* is synthesized in the neuroblasts, but at mitosis, it is asymmetrically localized to the daughter ganglion mother cells and excluded from the daughter neuroblasts. In ganglion mother cells, *prospero* is translocated to the nucleus and is probably involved in specification of ganglion mother cell fate (Spana and Doe, 1995; Hirata *et al.*, 1995; Knoblich *et al.*, 1995; Doe and Spana, 1995).

Only a few human homeobox genes are known to be expressed in the eye. We are searching for such genes in the hope of identifying the molecular basis of some human eye pathologies. In the present communication, we describe the isolation of the human Prox 1 gene, its exon–intron structure, its pattern of expression in some embryonic and adult human tissues, and its mapping to chromosome 1q32.2–q32.3.

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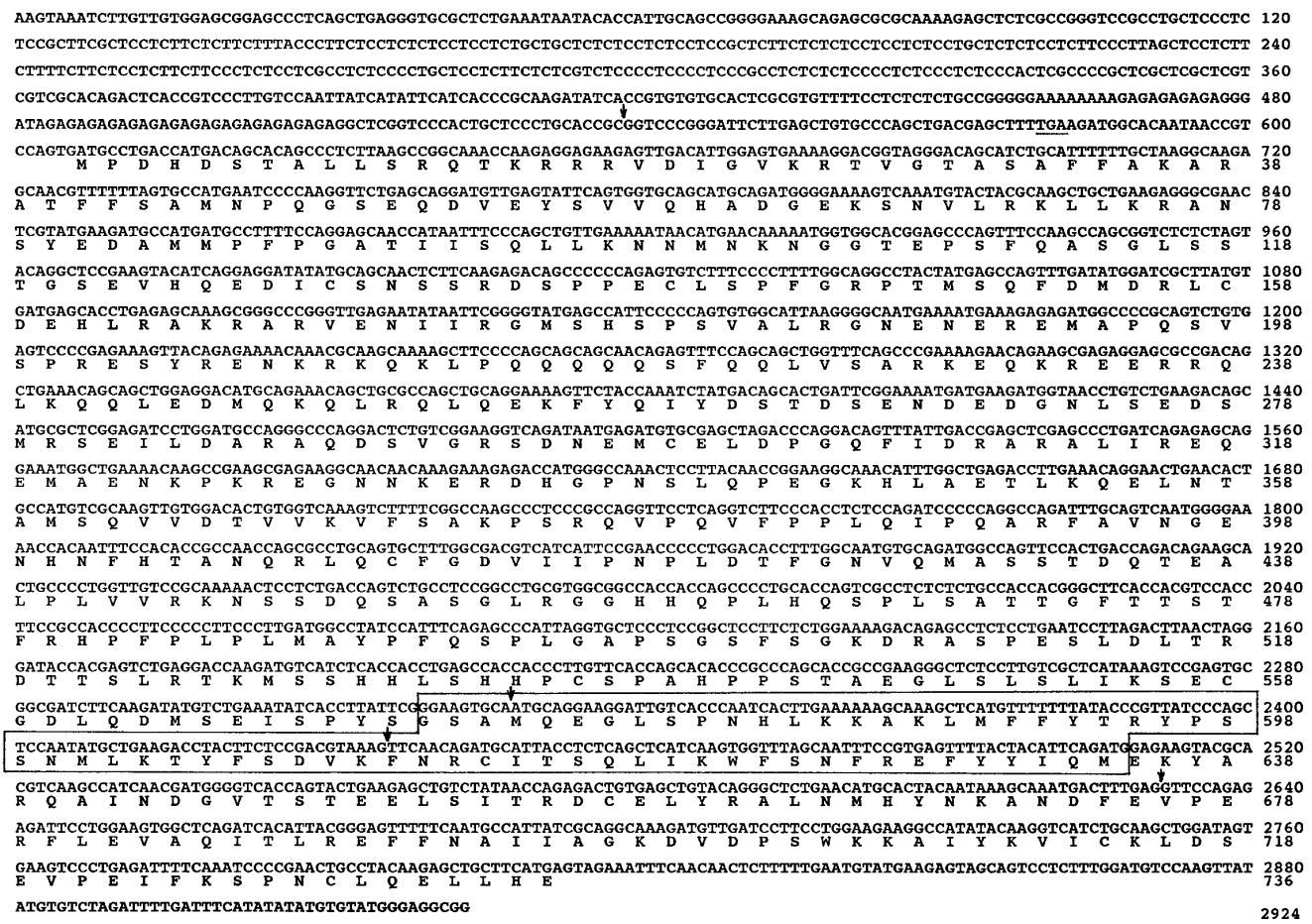

FIG. 1. Nucleotide sequence and deduced amino sequence of the human Prox 1 cDNA. The homeodomain is boxed. The stop codon preceding the initiator methionine is underlined. Positions of introns are shown by arrows. GenBank Accession No. U44060.

MATERIALS AND METHODS

Isolation of human Prox 1 λ phage and P1 genomic clones. A 517-bp PCR fragment generated from the chicken Prox 1 cDNA (positions 1649–2165; Tomarev *et al.*, 1996) was gel purified using GeneClean (BIO 101, La Jolla, CA), labeled with ^{32}P by the random primer method (labeling kit from Stratagene, La Jolla, CA), and used as a probe to screen a human λFixII genomic library (Stratagene). One million plaques were screened under moderately stringent conditions (hybridization in Quickhyb (Stratagene) and subsequent washing were performed at 55°C), and eight positive plaques were identified. Phage DNAs from positive plaques were isolated using the Promega (Madison, WI) λ DNA preparation kit, digested with different restriction enzymes, and analyzed by Southern blotting using the same PCR fragment that was used for library screening. Several *Eco*RI, *Hin*dIII, and *Bgl*II restriction fragments from the inserts of two overlapping clones (hybridizing and not hybridizing with the chicken PCR probe) were cloned into the pBlueScript plasmid vector (Stratagene) and sequenced. The nucleotide sequence obtained was used to design human Prox 1-specific PCR primers. They were (1) 5′-AAGACAGAGCCTCTCCTGAATC-3′ (5′ primer, positions 2123–2144 in Fig. 1) and (2) 5′-TTGCACTTCCCGAATAAGGTGAT-3′ (3′ primer, positions 2307–2329 in Fig. 1). These primers were used to screen a human P1 genomic library. This screening was performed as a service by Genome Systems, Inc. (St. Louis, MO). Three P1 clones (2746, 2747, and 2748) were identified and used in all subsequent experiments. DNA from the P1 clones was isolated as recommended by Genome Systems.

Isolation of Prox 1 cDNA clones. A 508-bp PCR fragment of the human Prox 1 cDNA (positions 1417–1924 in Fig. 1) was used to screen a λgt11 embryonic human brain cDNA library (Clontech, Palo Alto, CA). Ten million plaques were screened and approximately 500 hybridizing phages were identified. Phage DNAs from 5 of them were isolated using the polyethylene glycol precipitation method, subcloned into pBluescript, and sequenced. 5′ RACE reactions were performed using 5′-RACE-Ready embryonic human brain cDNA (Clontech), according to the manufacturer's instructions with the modifications described below. rTth DNA polymerase XL (GenAmp XL PCR Kit, Perkin–Elmer, Norwalk, CT) was used for PCR amplification, and the products were gel-purified and cloned into pCR-Script SK(+) using the conditions recommended by the manufacturer (Stratagene). The Prox 1 gene-specific primers were 5′-GAATGG-CTCATACCCCGAATTAT-3′ and 5′-GCGATCCATATCAAACTGG-CTC-3′ (antisense strand, positions 1120–1142 and 1053–1074, respectively; see Fig. 1).

DNA sequencing and analysis. DNA sequencing was carried out by the dideoxynucleotide chain termination method with ^{35}S-dATP (Sequenase, Version 2.0, Amersham). Nucleotide and amino acid sequences were analyzed using the GCG package (Genetics Computer Group, 1994). The intron–exon boundaries were determined by comparing cDNA and genomic sequences. The length of the introns was estimated by a combination of direct sequencing and PCR amplification using the corresponding P1 clones or recloned restriction fragments as templates.

Southern and Northern blotting. Five to ten micrograms of genomic DNA was digested with the indicated restriction enzymes, separated by electrophoresis in 0.8% agarose gels, transferred to Duralon UV filters (Stratagene), cross-linked by ultraviolet irradiation (Stratalinker, Stratagene), and hybridized with different ^{32}P-labeled PCR fragments in QuickHyb as recommended by the manufacturer (Stra-

tagene). Blots containing heterologous DNAs (see Fig. 3) were hybridized and washed under conditions of reduced stringency at 62°C. Poly(A)$^+$ RNA blots from adult and embryonic human tissues were purchased from Clontech. Total RNA was isolated from 26-week-old embryonic human lenses (a gift of Dr. J. Horwitz, University of California, Los Angeles) by the acidic guanidinium thiocyanate–phenol–chloroform extraction method (RNazol B, Tel-Test, Friendswood, TX). Ten micrograms of this RNA was separated by electrophoresis on a 1.2% agarose, 2.2 *M* formaldehyde gel, transferred to a Duralon UV membrane, and cross-linked. Hybridization was performed in QuickHyb under the conditions recommended by the manufacturer.

Mapping of the Prox 1 gene and isolation of YAC clones. Oligonucleotides (1) 5′-AAGACAGAGCCTCTCCTGAATC-3′ (5′ primer, positions 2123–2144, exon 2) and (3) 5′-TAGATATTGTGTAAACCCAACC-3′ (3′ primer, intron 2) were used to screen NIGMS human/rodent somatic cell hybrid mapping panel 2 (Drwinga *et al.*, 1993) by PCR of the somatic cell hybrid DNA. The human mega-YAC CEPH "B" library (Research Genetics, Huntsville, AL) was screened by PCR analysis of arrayed clone pools using primers 1 and 3. Positive clone 956-E-4 was selected for the presence of the YAC vector on AHC plates, and liquid cultures were prepared in YPD broth. Yeast DNA was prepared using standard protocols and analyzed first by PCR with STS ATC-P7501 primers (5′-AGATGATCAAACCACCTCCA-3′ and 5′-GTGCTTTGGCATATGGTAGG-3′) to confirm identity of the YAC 956-E-4 clone and then with primers 1 and 3 to confirm the presence of Prox 1 sequences. DNA from this positive clone together with human genomic DNA was analyzed by Southern blotting as described above.

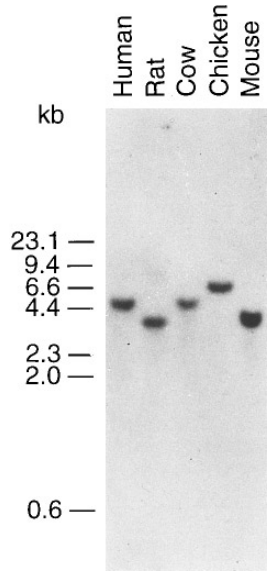

FIG. 3. Southern blot hybridization of the human Prox 1 gene. 8 μg of the indicated DNAs was digested with *Eco*RI, separated by electrophoresis on an 0.8% agarose gel, and hybridized with a ^{32}P-labeled 508-bp PCR fragment (positions 1417–1924) as described under Materials and Methods.

RESULTS

Isolation and Structural Characterization of the Human Prox 1 Gene

To isolate the human Prox 1 gene, we screened a human genomic library using a 517-bp PCR fragment corresponding to positions 528–700 of the amino acid sequence of the chicken Prox 1 cDNA (Tomarev *et al.*, 1996). This region is identical between chicken and mouse with the exception of a one amino acid change and includes the homeodomain and a portion of the *prospero* domain (Tomarev *et al.*, 1996). In preliminary experiments, it was shown that this chicken PCR fragment gave specific bands with human genomic DNA in Southern blot hybridization under conditions of moderate stringency (not shown). The DNA inserts of eight hybridizing phages were analyzed by Southern hybridization and sequencing. Comparison of the nucleotide sequences obtained with those of the chicken Prox 1 cDNA indicated that the isolated λ clones contained two exons of the human Prox 1 gene (exons II and III in Fig. 2). The complete human Prox 1 gene was isolated by screening a human P1 genomic library. Cloning and sequencing of overlapping restriction fragments from three overlapping P1 clones (see Fig. 2) in combination with the isolation and sequencing of cDNA clones from an embryonic human brain cDNA library and 5′ RACE clones obtained using embryonic human brain PCR-ready cDNA as a template (see Materials and Methods) led to the elucidation of a partial cDNA sequence for human Prox 1 (Fig. 1). Comparison of the cDNA and genomic sequences indicated that the Prox 1 gene consists of at least five exons spread over more than 42 kb of DNA (Fig. 2).

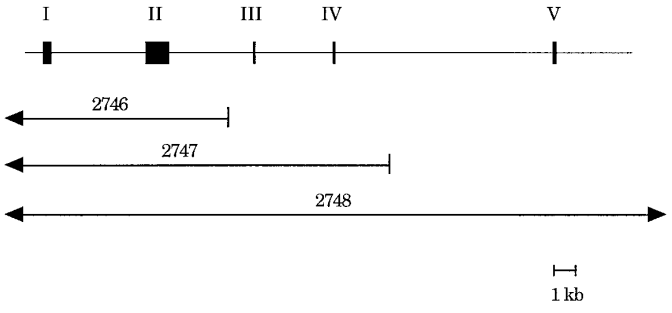

FIG. 2. Exon–intron structure of the human Prox 1 gene. Localization of the overlapping P1 clones used in this work is shown below.

The available sequence of the human Prox 1 cDNA is 2924 bp long. It contains an open reading frame of 2208 nt. The deduced amino acid sequence is 736 amino acids long with a calculated molecular mass of 83.2 kDa. A stop codon is localized 27 nt upstream of the first methionine codon in the reading frame exactly in the same position as in the chicken Prox 1 cDNA (Tomarev *et al.*, 1996), indicating that the coding region of this transcript is complete. The 5′ untranslated region of the longest clone obtained by 5′ RACE is 606 nt and is encoded by two exons. At present we do not know how much of the 5′ untranslated region of the Prox 1 mRNA is missing in our longest clone and whether there are any other exons upstream of the sequence we have designated as exon I. The 3′ untranslated region in the longest cDNA clone is only 107 nt and does not contain a typical polyadenylation signal, suggesting that it has been truncated during the cloning procedure.

Prox 1 Gene and Protein Are Conserved among Vertebrates

Comparison of the human and chicken Prox 1 nucleotide sequences has demonstrated that they are very conserved, having 94, 89, and 83% identity in the 5′ untranslated (part of exon II), coding, and 3′ untranslated regions, respectively. However, exon I of the human gene shows no clear similarity to the three identified alternatively spliced variants of the chicken Prox 1 cDNA (Banerjee-Basu, unpublished results). Exon I contains repetitive sequences that gave multiple matches with the noncoding sequences of many genes in the GenBank database. A human Prox 1 PCR fragment (exon II, positions 1417–1924) hybridized efficiently with genomic DNA from several different vertebrate species (Fig. 3). The presence of only one hybridizing fragment for each species indicates that there is only one Prox 1 gene in vertebrates. Overall, the human and chicken Prox 1 proteins show 94% identity. In the region of the homeodomain and prospero domain, the two proteins are identical. Human Prox 1 shows 65 and 56% identity with the homeo- and prosperodomains, respectively, of *Drosophila prospero.* Upstream of the homeodomain, there are two short regions of 35 and 50% identity between human Prox 1 (positions 216–269 and 348–371) and *Drosophila prospero* (positions 523–576 and 838–861, respectively) as observed before for chicken Prox 1 (Tomarev *et al.*, 1996). It is interesting to note that this second region of identity is localized just upstream of the sequence (positions 871–902) that was proposed to be essential for the asymmetric distribution of *Drosophila prospero* (Hirata *et al.*, 1995). No other similarities were detected between the upstream sequence of human Prox 1 and other proteins in the GenBank database.

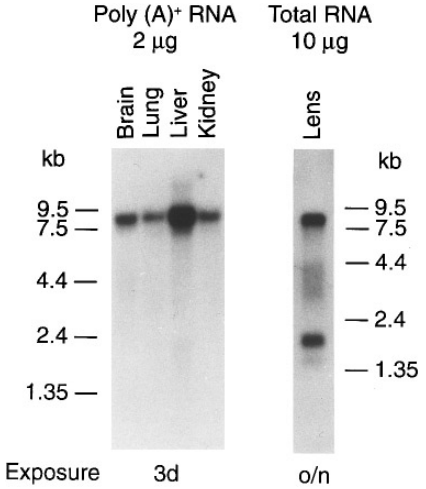

FIG. 4. Expression of the human Prox 1 gene in different embryonic tissues. A Northern blot containing 2 μg of poly(A)$^+$ RNA from human brain, lung, liver, and kidney was purchased from Clontech. 10 μg of total RNA from human embryonic lens was separated by agarose electrophoresis as described under Materials and Methods. Blots were hybridized with the same probe as in Fig. 3.

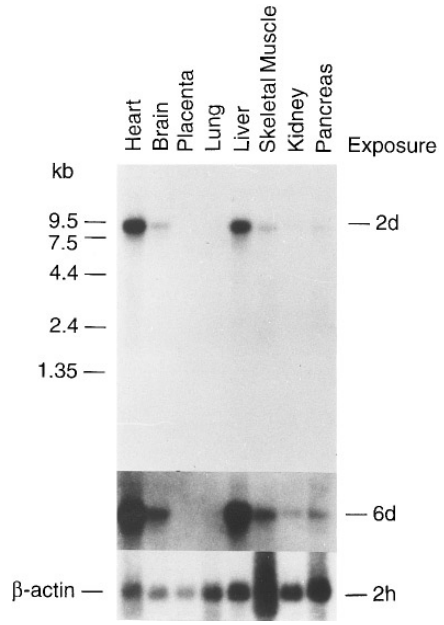

FIG. 5. Expression of the human Prox 1 gene in adult tissues. A Northern blot containing 2 μg of poly(A)$^+$ RNA from human tissues was purchased from Clontech. The blot was hybridized with the same probe as in Fig. 3. A human β-actin probe (Clontech) was used as a positive control.

Expression Pattern of the Human Prox 1 Gene

The size and distribution of the human Prox 1 mRNA were investigated by Northern blot hybridization. Prox 1 mRNA was detected in all five human embryonic tissues analyzed: lens, brain, lung, liver, and kidney (Fig. 4). Lens demonstrated the highest level of Prox 1 mRNA. Among the adult tissues analyzed, Prox 1 mRNA was more abundant in heart and liver than in brain, skeletal muscle, kidney, and pancreas and was not detected in placenta and lung (Fig. 5). In all tissues but lens, the Prox 1 cDNA hybridized to a single band with a length of around 8 kb. In the embryonic lens, an additional component with a length of 2 kb was present. Since there is only one Prox 1 gene in the human genome, these data indicate that the Prox 1 gene may be alternatively spliced.

Mapping of the Human Prox 1 Gene

The human Prox 1 gene was first mapped to chromosome 1 by PCR analysis of a panel of somatic cell hybrids (Drwinga *et al.*, 1993). Several combinations of oligonucleotides were tested with human, mouse, and hamster genomic DNA before oligonucleotides (1) and (3) were chosen (see Materials and Methods). This pair gave a single 283-bp PCR fragment with human DNA but not with mouse or hamster DNA. These same primers were used to screen the human mega-YAC CEPH B library, and YAC clone 956-E-4 was identified. Yeast DNA from this clone was isolated and analyzed both by PCR and Southern blot hybridization. The results demonstrated (Fig. 6) that YAC 956-E-4 indeed con-

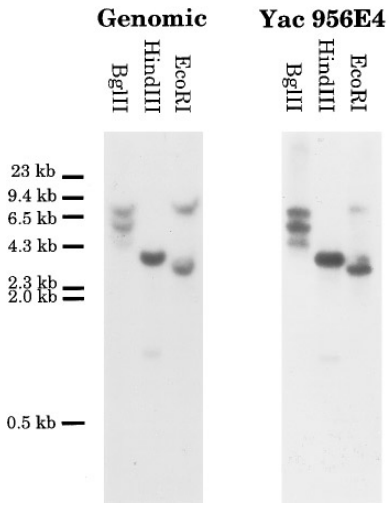

FIG. 6. Southern blot hybridization of the human Prox 1 gene. Human DNA and yeast DNA (mega-YAC clone 956-E-4) were digested with the indicated restriction enzymes, separated by electrophoresis on a 1% agarose gel, and hybridized with the ^{32}P-labeled PCR fragment (positions 2240–2862) as described under Materials and Methods.

tained the human Prox 1 gene. Using the YACSR program (M. Polymeropoulos, unpublished results), we found YAC 956-E-4 to be a part of the chromosome 1 contig WC-1179 that contains YACs 684-F-7, 750-E-4, 958-E-1, and 967-G-10. This contig was recently mapped to human chromosome 1q32.2–q32.3 (see Chumakov *et al.,* 1995).

DISCUSSION

In the present work, we isolated and characterized the human gene encoding the homeodomain protein Prox 1. This gene consists of at least 5 exons and 4 introns and encodes a protein with a calculated molecular mass of 83.2 kDa. The characterized portion of the human Prox 1 gene most probably contains the entire protein coding region since a stop codon was identified upstream of the potential initiator methionine codon (see Fig. 1). The highly homologous chicken Prox 1 protein has an apparent molecular mass of about 80–82 kDa according to Western immunoblotting data (Tomarev *et al.,* 1996). However, since the length of the longest human Prox 1 mRNA by Northern blot hybridization is about 8 kb and we cloned only 2924 bp of its cDNA, significant amounts of the 5′ and 3′ untranslated regions must be missing from our clones. Our attempts to isolate longer clones and obtain additional sequence by 5′ RACE were not successful. The known chicken Prox 1 cDNA (3322 bp), the only available nucleotide sequence of Prox 1 in vertebrates, has 1043 nt of 3′ untranslated region that may also be incomplete. In the 5′ untranslated region corresponding to exon I in the human sequence, we could not find any similarities with the available 5′ untranslated region of the chicken Prox 1 cDNA (Banerjee-Basu, unpublished results). Since no information is available on the exon–intron structure of the Prox 1 gene in other vertebrates, we cannot eliminate the possibility that there are other exons in both the 5′ and 3′ the untranslated regions of the human Prox 1 gene, making it significantly longer than 40 kb. There is some information on the exon–intron structure of the *Caenorhabditis elegans prospero* gene. In the region where the *C. elegans prospero* and human Prox 1 genes are homologous, i.e., regions encoding the homeodomain and C-terminus, there are two introns in the former (Wilson *et al.,* 1994) and three introns in the latter. The position of only one intron (intron 4 in the human Prox 1 gene, Fig. 2) is conserved in both genes.

There is only one Prox 1 gene in vertebrates including human; therefore the presence of two hybridizing bands in lens RNA indicates that the Prox 1 gene is alternatively spliced. A similar situation was observed for the chicken Prox 1 gene in which two abundant bands with lengths of 8 and 2 kb were observed in the lens (Tomarev *et al.,* 1996). The 2-kb mRNA is too short to encode the full-length 83-kDa protein, and further studies are necessary to determine this alternatively spliced Prox 1 mRNA and encoded protein. It is interesting to note that one of the chicken Prox 1 cDNA clones we identified lacked the sequence corresponding to exon IV and three other cDNA clones had different sequences in the region corresponding to exon I of the human Prox 1 gene (Banerjee-Basu, unpublished results).

Although information on the expression pattern of human Prox 1 is rather limited, the present data correlate well with the pattern of Prox 1 expression in the chicken and mouse (Oliver *et al.,* 1993; Tomarev *et al.,* 1996). In the chicken, Prox 1 is actively expressed in the developing lens starting from the early lens placode, in horizontal cells of the retina, and in the developing liver and pancreas. The conserved structure and expression pattern of the Prox 1 gene imply that it may play the same roles in human as in other vertebrates.

The human Prox 1 gene was mapped to the q32.2–q32.3 region of human chromosome 1. Usher syndrome type II, which is associated with hearing loss and retinitis pigmentosa, mapped close to this area between markers D1S237 and D1S229 (Kimberling *et al.,* 1995). The genetic linkage of genes located in this general vicinity on human chromosome 1 has been conserved in mammalian evolution as two large blocks of synteny residing on mouse chromosome 1 around positions 65 and 115 (see Seldin, 1994 for summary). The mouse mutation *rd3,* a primary retinal degeneration syndrome, was identified and mapped to position 111 of mouse chromosome 1 (Chang *et al.,* 1993). Prox 1 can be considered a candidate gene for human Usher syndrome type II and mouse *rd3,* but further studies are necessary to elucidate any connection between Prox 1 and these or any other diseases.

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