



Plus/Minus Screening of Rabbit Corneal Endothelial cDNA Library

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Abstract: Plus/minus screening of the rabbit corneal cDNA library was performed using corneal and iris RNA as probes. Thirteen clones were isolated: three ferritin H-chains, a NADH-ubiquinone oxidoreductase B22 subunit, an alpha 1 type VIII collagen, a 25 KDa FKBP-506 binding protein (FKBP25), a thrombospondin 2, and six unknown clones. Although proteins translocated from these isolated mRNA are not corneal specific, they play an important role in the cornea. None of the isolated known mRNAs maps to chromosome 1, 16, or 20. These clones, thrombospondin excepted, were not observed in the high frequency clones in the profile of the aortic endothelial cDNA library. **Jpn J Ophthalmol 1997;41:370-375** © 1997 Japanese Ophthalmological Society

Key Words: cDNA, cornea, ferritin, NADH-ubiquinone oxidoreductase, thrombospondin.

Introduction

The cornea is highly differentiated tissue which plays important roles in the eye. It is composed of five layers: the endothelium, the inner most layer, is where the barrier and pump functions are located. The integrity of the cornea is maintained by the barrier and active transport functions of the endothelium. Although the human corneal endothelial cell is not regenerative, the rabbit corneal endothelial cell is regenerative during wound healing.¹ The mechanism of these important functions in the corneal endothelial cell has not been sufficiently investigated by either biochemical or molecular biological methods.

There are a few studies on cloning from the corneal cDNA library. Since the cellular component is poor in corneal tissue, only a small amount of mRNA can be obtained from it. As a result, a good corneal cDNA library was hardly constructed. Rabbit K3 keratin cDNA was isolated from a corneal epithelial cell cDNA library,² rabbit decorin cDNA from a corneal cDNA library and a corneal stromal (keratocyte) library,³ chick decorin cDNA from a chick corneal cDNA library,⁴ chick lumican cDNA from a chick

corneal cDNA library,⁵ bovine lumican from a bovine corneal cDNA library,⁶ several kinds of collagens from a rabbit corneal endothelial cDNA library.^{7,8,9} These clones undoubtedly have important functions in the cornea.

There are two strategies to isolate important cDNA clones which express in specific tissues. One is the expression profile study¹⁰ and the other is the plus/minus screening approach.¹¹ Recent investigation employing the human corneal epithelial cDNA library showed a gene expression profile of human corneal epithelium.¹² Apolipoprotein J was synthesized and highly expressed in the corneal epithelium and localized in the ocular surface interface.¹³ To obtain corneal endothelial cDNA, we performed a plus/minus screening of the rabbit corneal endothelial cDNA library using corneal and iris mRNA as probes. We have already characterized and reported on the isolated clone, rabbit 25 KDa FKBP-506 binding protein (FKBP25).¹⁴ This study is a characterization of the other isolated clones.

Materials and Methods

Plus/Minus Screening of Rabbit Corneal Endothelial cDNA Library

The housing and use of animals conformed to the Declaration of Helsinki of the World Medical Asso-

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ciation. All tissue samples used in the experiments reported here were from adult rabbits; medical examinations of the animals revealed no diseases. Rabbits were sacrificed by an overdose of intravenous pentobarbital anesthesia, and organs were removed. Corneas and irises were dissected from surrounding tissues and homogenized, using a power homogenizer (PhycotronTM, Nichi-On Medical Supply, Funabashi, Chiba) in TRIzolTM Reagent (Life Technologies, Gaithersburg, MD, USA) 1 mL per 50–100 mg of each tissue. Total RNA was obtained using the isolation method developed by Chomczynski and Sacchi.¹⁵

Rabbit corneal endothelial cDNA library⁷ was obtained from the Department of Molecular Biology and Biochemistry, Okayama University Medical School. Plus/minus screening¹¹ was performed in 1000 clones from the library. The phage was plated and the phage DNA was transferred onto nylon membrane (DuPont/NEN Research Products, Boston, MA, USA). The ³²P-labeled corneal and iris cDNA probes were prepared and hybridized with the membrane at 65°C. Clones which hybridized with corneal cDNA probe but did not hybridize with iris cDNA probe were isolated.

DNA Preparation and Sequence Analysis of Isolated Clones

Isolation and purification of phage DNA were performed according to the standard method using the SephaglasTM PhagePrep Kit (Pharmacia Biotech, Milwaukee, WI, USA).^{16,17} The insert of the phage DNA was amplified by polymerase chain reaction (PCR) using the primers and the conditions described previously.¹⁴ Amplification was performed in a thermal cycler model PJ2000 (Perkin Elmer Cetus, Norwalk, CT, USA). The PCR product was directly sequenced by the dideoxy chain termination method¹⁸ using an automated sequencer¹⁹ (model 373A; Advanced Biotechnologies, Columbia, MD, USA) and synthetic nucleotide as primers. Sequences were edited and compared to the EMBL and Genbank databases.^{20,21}

RT-PCR Analysis of Isolated Clones

Reverse transcription-polymerase chain reaction (RT-PCR) analysis was performed in tissue samples from conjunctiva, cornea, iris, retina, cerebrum, cerebellum, cardiac muscle, lung, liver and kidney. Each first-strand cDNA for PCR was synthesized from each mRNA in each of the above total RNA by using the SUPERSRIPTTM preamplification system²²

(Life Technologies). The condition for the cDNA synthesis was described previously.¹⁴ Each cDNA was then used for amplification of each specific primer mixture for each clone with α -actin as an internal standard cDNA sequence in RT-PCR. Upstream and downstream PCR primers for α -actin were used as previously reported.²³ For each experiment involving PCR, separate tubes containing all reagents except cDNA were used as negative controls for detecting nonspecific amplification products. Amplification was performed in a thermal cycler under conditions described earlier.¹⁴ Each PCR product was electrophoretically fractionated on a 2% agarose gel containing ethidium bromide visualized by ultraviolet light, and photodocumented using PolaroidTM photography. The band intensities on the agarose gel were measured by a densitometer and normalized to the relative intensity of the α -actin band.

Results

Plus/Minus Screening of Rabbit Corneal Endothelial cDNA Library

Figure 1 shows the results of the plus/minus screening. Clones 4a, 4b, and 11a hybridized strongly with the corneal cDNA probe; however, only faintly with the iris probe. Figure 2 shows the results of 2% agarose gel electrophoresis of the insert cDNAs amplified by PCR using the primers corresponding to the lambda phage DNA sequence. The insert DNAs ranged from 200 to 2000 base pair. Longest cDNA sequences named clones 1 and 2 are not shown here.

DNA Preparation and Sequence Analysis of Isolated Clones

We edited the DNA sequences of the clones and compared them with previously reported databases. Thirteen clones were isolated: three ferritin H-chains, a NADH-ubiquinone oxidoreductase B22 subunit, an alpha 1 type VIII collagen, a FKBP25, a thrombospondin 2 and six unknown clones (Table 1). The 3' nucleotide and deduced amino acid sequence of rabbit NADH-ubiquinone oxidoreductase B22 subunit compared with bovine²⁴ and human²⁵ sequences are shown in Figure 3.

RT-PCR Analysis of Isolated Clones

The expression of mRNA was observed in all tissues examined. Although the RT-PCR analysis showed a relatively high expression of clones in the cornea compared to the iris, we could not identify corneal specific clones.

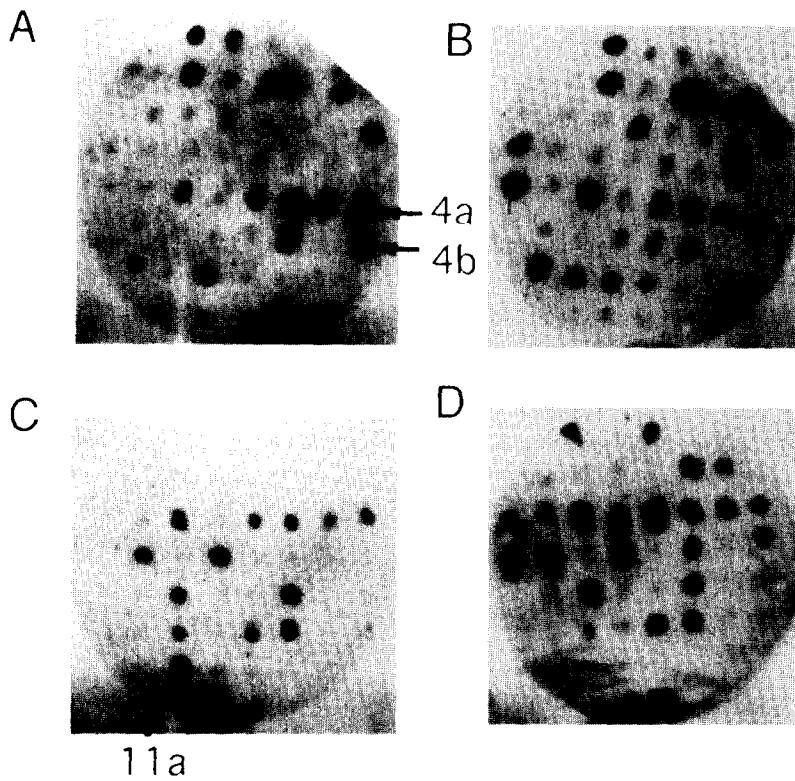


Figure 1. Examples of results of plus/minus screening using rabbit corneal endothelial cDNA library. (A, C) Blots hybridized with corneal mRNA probe. (B, D) Blots hybridized with iris mRNA probe. (A, B) and (C, D) are the same blots. Clones hybridized with corneal probe were isolated much more than those with iris probe.

Discussion

We have reported on the rabbit FKBP25 cDNA and its high expression in corneal epithelium.¹⁴ Other isolated clones also have important functions in the cornea. We isolated three ferritin H-chains. Ferritin, an iron protein, plays a central role in recycling iron for the synthesis of heme and other pro-

teins. Iron deposition in the cornea occurs secondarily in a number of clinical settings. Freischer's ring, a deposit of hemosiderin is seen in the cornea of patients with keratoconus.²⁶ The pathogenesis of corneal iron lines is still unclear. Ferritin may play an important role not only in heme protein synthesis, but also in the cornea which has no vascular system. Pathogenic change may cause disorder of the iron

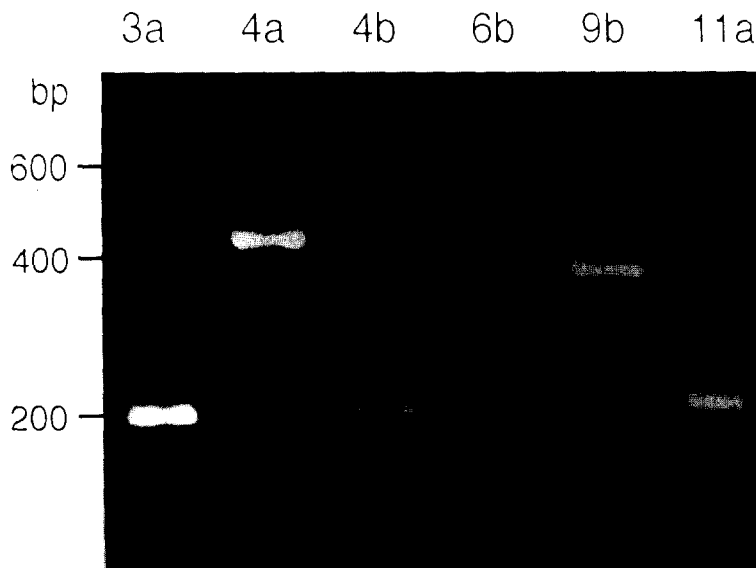


Figure 2. Results of 2% agarose gel electrophoresis of PCR amplified DNAs encompassing insert of λ gt10 clones. 3a, 4a, 4b, 6b, 9b, and 11a indicate examples of clone. bp, base pair.

Table 1. Clones Isolated by Plus/Minus Screening

Products	Number
Ferritin, H-chain	3
FK506/rapamycin binding protein 25	1 ^a
NADH-ubiquinone oxidoreductase B22 subunit	1
Thrombospondin 2	1
Alpha 1 type VIII collagen	1
Unknown	6

^aPartial sequence of rabbit FK506/rapamycin binding protein 25 has already been reported.¹⁴

metabolism, resulting in Fleischer's ring. Ferritin is composed of only two types of subunit H- and L-chains. The relative amounts of these subunits vary by tissue and by species.^{27,28} For example, human liver ferritin composed of 95% L-chain and human heart ferritin is composed of 60% H-chain. Our isolated clones were entirely H-chain. The high frequency of H-chain and low frequency of L-chain in the expression profile of the human corneal epithe-

lial cell library¹² indicate that corneal isoferritin may be mainly compose of H-chain ferritin.

Since clone 4a shows a high homology with the bovine NADH-ubiquinone oxidoreductase B22 subunit²⁴ (Figure 3), it should be the rabbit NADH-ubiquinone oxidoreductase subunit B22. As the corneal endothelium contains a large amount of mitochondria, the mitochondrial enzymes should play an important role in the corneal endothelium. ATPs produced by mitochondrial enzymes are critical for endothelial pump function.

We also isolated the alpha 1 type VIII collagen. The sequence of alpha 1 type VIII collagen was determined using the same rabbit corneal endothelial cDNA library.⁷ Type VIII collagen was originally isolated from the culture medium of bovine aortic endothelial cells and designated as endothelial cell (EC) collagen.²⁹ It is also produced by corneal endothelial cells.³⁰ Tamura and co-workers³¹ investigated the tissue distribution of collagen VIII in human adult and fetal eyes immunohistochemically and showed that it is distributed in Descemet's mem-

A.

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Rabbit : CAGGAGGAAACCCCACCCGGTGGTCCTAAGACTGAAGCTTTGCCCCCTGCCCGGAAGGAA
Bovine : CAGGAGGAAACCCCAGTTGGTGGTCCCCGAAGCTTTGCCCCAGCCCGAAAGCAA
Human  : CAGGAGGAAACGCCACCTGGTGGTCCTTAACTGAAGCTTTGCCCCCTGCCCGTAAGGAA
***** **      *****      ***** ***** ** **
Rabbit : GCGATTGCCCCACTGTGGTGGCACATTGTGACCAGGCCCGGGAGCGGCCACGTAG
Bovine : GGTGACTTCCCCCTCTGTGGTGGCATATCGTGACCAGACCCCGGGAGCGGCCATGTAG
Human  : GGTGATTGCCCCACTGTGGTGGTATATTGTGACCAGACCCCGGGAGCGGCCATGTAG
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B.

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Rabbit : QEETPPGGPKTEALPPARKEGDLPLLWWHIVTRPRERPT
Bovine : QEETPVGGPRTEALPPARKQGDLPPLLWWHIVTRPRERPM
Human  : QEETPPGGPLTEALPPARKEGDLPLLWYIVTRPRERPM
***** ** ***** ***** *****
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Figure 3. Comparison of (A) nucleotide and (B) deduced amino acid sequence among bovine, rabbit, and human NADH-ubiquinone oxidoreductase B22 subunit. Both nucleotide and amino acid sequences represent 3' coding and C-terminal region respectively. Asterisks indicate homologous nucleotides and residues. Underline indicates stop codon.

brane of the cornea. Type VIII collagen is one of the most important collagens in endothelial cells.

Thrombospondin (TSP) is a constituent of the extracellular matrix and plays a role analogous to fibronectin and laminin in cell attachment, differentiation, and metastasis. Five family (TSP-1 to TSP-5) members have been isolated. TSP-1 and TSP-2 are similar homotrimeric molecules; recent investigation showed that TSP-1 is a potent inhibitor of the migration, mitogenesis and sprouting of cultured endothelial cells in vitro and of neovascularization in vivo.^{32,33} It is observed at a high frequency in the expression profile of the aortic endothelial cell library, indicating it is a very important protein in endothelial cells. Fibronectin is the most frequently observed clone in the expression profile of the human aorta endothelial library.³⁴ The clones isolated in this study, thrombospondin excepted, were not observed among the high frequency clones in the profile of the aortic endothelium library.

There are many kinds of corneal dystrophies. The causative genes for Meesmann's, granular, Avellino, lattice type I and II and Reis-Bückler corneal dystrophy were determined.³⁵⁻³⁸ Macular corneal dystrophy, caused by a systemic disorder of keratan sulfate metabolism, was recently mapped to chromosome 16q.³⁹ Schnyder's crystalline corneal dystrophy was recently mapped to chromosome 1.⁴⁰ Posterior polymorphous dystrophy and congenital hereditary endothelial dystrophy were mapped to chromosome 20.^{41,42} Candidate gene approach has succeeded in finding the causative gene for retinal dystrophies⁴³ for several reasons: (1) many candidate genes have been isolated and sequenced, (2) many DNA samples from retinal dystrophy patients have been obtained, (3) screening procedure including single strand conformation polymorphism procedure for genetic defects has been developed, and (4) it became easy to screen the visual functions of the patients with retinal dystrophy using electrophysiological tests. Points (2) and (3) can be applied to corneal dystrophy; as for (4), patients with corneal dystrophy can be easily screened by slit-lamp examination. However, many candidate genes for corneal dystrophy are required. In our isolated clones, the ferritin H-chain mapped to chromosome 11;⁴⁴ NADH-ubiquinone oxidoreductase B22 subunit, to chromosome 8;²⁵ thrombospondin 2, to 6q27.⁴⁵ None of the isolated known clones map to chromosomes 1, 16 or 20. Although the applicability of this approach for corneal dystrophy is obscure, we plan further study including the chromosomal localization of the unknown clones.

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