Biochemical and Biophysical Research Communications **276,** 12–15 (2000)
doi:10.1006/bbrc.2000.3422, available online at http://www.idealibrary.com on IDEAL®

A Novel Gene (Retinovin) Expressed Selectively in the Early Stage of Chick Retinal Development

Masako Itami, Toshihiko Matsuo,[1] and Hiroshi Ohtsuki
Department of Ophthalmology, Okayama University Medical School, Okayama City 700-8558, Japan

Received August 4, 2000

To understand molecular mechanisms of retinal development, genes expressed selectively only in the early stage of retinal development were isolated by subtractive hybridization based on suppression polymerase chain reaction. The retina has no layered structure in 7-day chick embryos, in contrast with the fully developed multilayered structure of neurons in 15-day embryos. The subtraction between cDNA derived from retinal tissues at these different stages, followed by repeat rounds of 5′-RACE (rapid amplification of cDNA ends) and 3′-RACE, led to isolation of a novel gene with an open reading frame encoding a putative protein with 753 amino acids. Its specific expression in the 7-day embryonic retina was confirmed by Northern blot analysis. The gene, named "retinovin," would be used as a marker for identifying retinal stem cells present at the early stage of retinal development. © 2000 Academic Press

Key Words: **retina; development; subtractive hybridization; chick; cDNA; retinovin.**

The retina is part of the central nervous system and provides a simplified model to study neuronal development. During embryogenesis, the outer plate of the optic cup remains a monolayer of cells and begins to have pigments to form the retinal pigment epithelium. In contrast, its inner plate, also a monolayer of cells at the initial phase, becomes a multilayered structure of neurons to develop the retina. Genes responsible for this process of retinal development have not been well characterized (1).

The retina in the early stage of development has a large number of multipotent stem cells which are capable of generating both neurons and glial cells (2, 3). To identify these stem cells will open a new strategy to direct the retina to regenerate from stem cells in the case of retinal degenerative diseases. However, at present, no marker for identifying stem cells in the retina is available. Chick embryos have been used as a model for studying development because their embryonic stages are precisely determined and they are easily manipulated (4). In this study, we used suppression subtractive hybridization to isolate genes which were expressed selectively only in the early stage of chick retinal development.

MATERIALS AND METHODS

Isolation of mRNA and subtraction. The retina was isolated from 7-day and 15-day chick embryos (Fig. 1). In brief, embryos at either stage were taken out from a small opening of the egg shell. The eyes were enucleated and cut at the midperiphery of the globe, and the anterior halves were removed together with the vitreous gel. The retina was peeled off easily from the eye cup (5). Poly(A)+ RNA was isolated (Micro-FastTrack mRNA Isolation Kit, Invitrogen, Carlsbad, CA), and cDNA was synthesized. The subtraction of 15-day embryonic retinal cDNA from 7-day embryonic retinal cDNA was done with a suppression polymerase chain reaction (PCR)-based method (PCR-Select cDNA Subtraction Kit, Clontech Laboratories, Palo Alto, CA). Briefly, 7-day retinal cDNA and 15-day retinal cDNA were digested with a restriction enzyme, RsaI, to obtain shorter, blunt-ended cDNA. Two different adaptors (adaptor 1 and adaptor 2) were then ligated to 5′ end of each strand of 7-day retinal cDNA after the digestion. The adaptor 1-ligated 7-day retinal cDNA and adaptor 2-ligated cDNA were separately hybridized at 68°C for eight h with an excess of 15-day retinal cDNA after denaturation at 98°C for 1.5 min. The two primary hybridization samples were then mixed together without denaturation and hybridized at 68°C overnight with an excess of denatured 15-day retinal cDNA.

Differentially expressed sequences in 7-day retinal cDNA were amplified by two rounds of PCR. The first PCR amplification was based on suppression PCR to amplify only cDNA with different adaptors at both ends, which were further enriched by the second PCR amplification with nested primers (6, 7).

Isolation and sequencing of 7-day retina-specific cDNA clones and Northern blot analysis. The 7-day retina-specific sequences amplified by PCR were cloned into a pCR-TOPO vector (TOPO TA Cloning Kit, Invitrogen). Plasmids were isolated with Qiagen Plasmid Mini kit (Qiagen, Hilden, Germany) and then sequenced with ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit and ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems, Foster City, CA) using primers, pUC/M13 primers, forward and reverse (Promega, Madison, WI).

GenBank Accession No. AF242552.

[1] To whom correspondence should be addressed at Department of Ophthalmology, Okayama University Medical School, 2-5-1 Shikata-cho, Okayama City 700-8558, Japan. Fax: +81-86-222-5059. E-mail: matsuot@cc.okayama-u.ac.jp.

0006-291X/00 $35.00

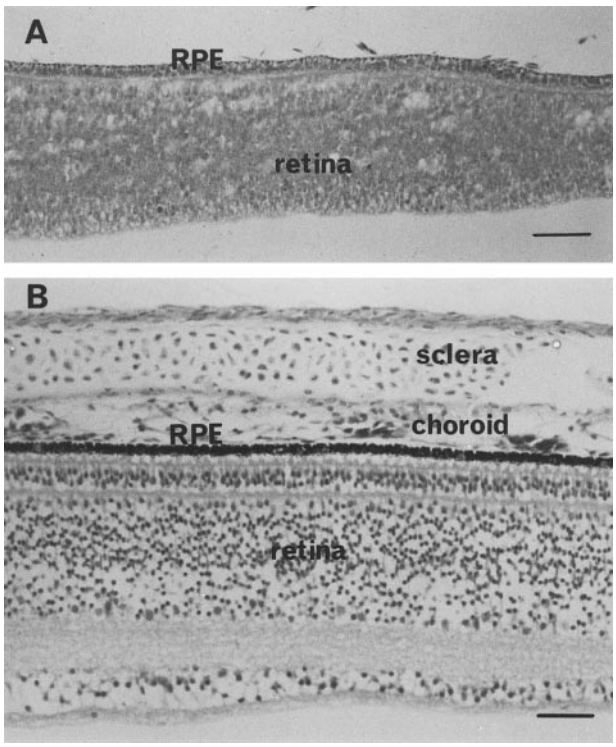

FIG. 1. Photomicrographs of the retina in a 7-day chick embryo (A) and a 15-day embryo (B). The retina becomes thick with cell proliferation but shows no stratification at 7 days (A), in contrast with fully developed multilayers of neurons at 15 days (B). RPE, retinal pigment epithelium. Bar, 50 μm.

Northern blot analysis was first done with dot blot. Isolated cDNA clones were labeled with alkaline phosphatase (AlkPhos Direct, Amersham Life Science, Buckinghamshire, England) and hybridized in each well of BIO-DOT Microfiltration Apparatus (Bio-Rad Laboratories, Richmond, CA) with mRNA which was isolated either from the 7-day or 15-day chick embryonic retina, and blotted on a sheet of nylon membrane (Hybond N+, Amersham). Signals were detected with chemiluminiscence (CDP-Star Detection Reagent, Amersham) on Hyperfilm MP (Amersham).

5′-RACE (rapid amplification of cDNA ends) and 3′-RACE. The procedure was done with Marathon cDNA Amplification Kit (Clontech). In brief, the first strand was synthesized with an oligo-dT primer or one of two gene-specific primers, followed by second strand synthesis. The adaptor was then ligated to both ends of the cDNA. After denaturation at 94°C for one minute, PCR was done with TaKaRa LA Taq polymerase (Takara Shuzo, Otsu, Japan) in the condition of 20 or 25 cycles of denaturation at 94°C for 30 sec, annealing at 60 or 65°C for 30 sec, and extension at 68°C for 5 min, followed by the final extension at 68°C for 7 min. The PCR-amplified sequences were cloned into a vector, pCR-TOPO vector, and sequenced.

Seven-day retina-derived and 15-day retina-derived mRNA, mixed with RNA Sample Loading Buffer (5 Prime – 3 Prime Inc., Boulder, CO), were separated with Mops buffer on a 1% agarose mini-gel containing 5.4% formaldehyde, and transferred by alkali blotting to a nylon membrane (Hybond N+) with Vacuum Blotting Unit and Pump (2016 VACUGENE, LKB Bromma). The blot was hybridized with an alkaline phosphatase-labeled cDNA fragment located within the coding region. Northern blot analysis was also done with mRNA isolated from the brain of 7-day and 15-day embryos.

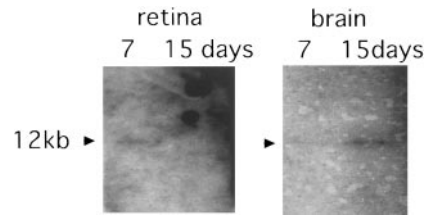

FIG. 2. Northern blot analysis. A new gene (retinovin) is expressed only in the retina of a 7-day embryo, but not in that of a 15-day embryo. In contrast, the gene is expressed in the brain of a 15-chick embryo, but not in that of a 7-day embryo.

RESULTS AND DISCUSSION

After subtractive hybridization with suppression PCR, a DNA smear extending from about 300 to 2000 base pairs (bp) was observed on 3% agarose gel electrophoresis. The smear DNA was cloned into a plasmid vector, and 178 plasmid clones with an insert DNA ranging from about 200 to 1200 bp in length were obtained. Of these clones, the insert of 88 clones were analyzed by dot-blot Northern hybridization, and four clones were found to be expressed selectively in the 7-day embryonic retina, compared with the 15-day embryonic retina. The insert of these four clones was then used as a probe in Northern blot analysis. A message, detected at about 12 kb by one clone (clone 60), was expressed selectively in the 7-day embryonic retina, but not in the 15-day embryonic retina (Fig. 2). After subtracting the adaptor sequence at both ends, the insert of this clone was 498 bp in length, and its sequence showed no matching in GenBank search. Messages detected by the other three clones were equally expressed in both the 7-day and 15-day embryonic retina (data not shown).

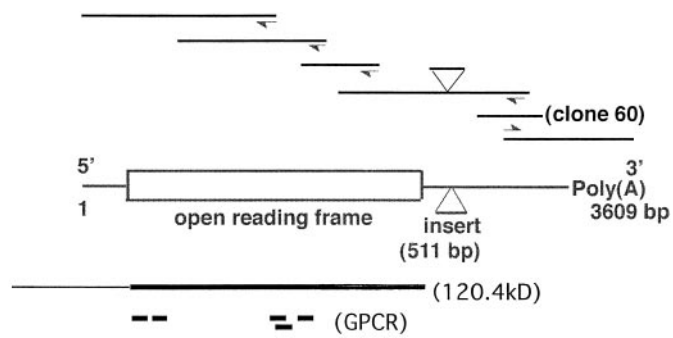

FIG. 3. Strategy to isolate the whole coding region of a new gene (retinovin) and its schematic structure. Several rounds of 5′-RACE and 3′-RACE were done with gene-specific primers (arrows) to extend from the initially isolated fragment (clone 60). The 3′ untranslated region contains a 511-bp insert. A region (thick line) showing homology with a human 120.4 kD protein of unknown function (GenBank Accession No. X59131) is indicated (120.4 kD). Also indicated are five domains of rhodopsin-like G protein-coupled receptor (GPCR) superfamily signature.

```
301' FAEIETCLNEVRDEIFISLQPQLRCTLGDMESPVFAFPLLLKLETHIEKLFLYSFSWDFE

  1"                                                    MLRNSSRIHFHGSLN

361' CSQCGHQYQNRHMKSLVTFTNVIPEWHPLNAAHFGPCNNCNSKSQIRKMVLEKVSPIFML
       *..* .* .*.*.****.**.******.* .*****...** *.*.****..*.*.
 16" ACGVGYKYLDRCRKTLTTFTNIIPDWHPLNAVHVAPCNNCSDRSQRRQMILEKVPSILMF

421' HFVEGLPQNDLQHYAFHFEGCLYQITSVIQYRAN-NHFITWILDADGSWLECDDXKGPCS
     ** ****.*.*. *.*..*. .********* .. .**.** *..**.******.***..
 76" HFQEGLPHNKLKSYSFQLEEDVYQITSVIQYAVHKKHFVTWSLNPDGTWLECDDLKGPYC

480' ERHKKFEVPASEIHIVIWERKISQVTDKEAACLPLKKTNDQHALSNEKPVSLTSCSVGDA
      ***.****..********.* *........  . *...*  . . .   ..  *. .**
136" RRHKRFEVPPTEIHIVIWEKKASRMPEEQDSQYQSKNIEDSSTNNVQLKSTVLHCGFDDA

540' ASAETASVTHPKDISVAPRT---LSQDTAVTHGDHLLSGPKGLVDNILPLTLEETIQKTA
     ...  *.  . ..... ..   ..... .. .    ...***..**  *  ..  *.
196" VDNMLAEDHQEDTVRIPDKKQQRVAEGESSARCGLENLARDGLVTQILEETPLDSEGKSM

597' SVSQLNSEAFLLENKPVAENTGILKTNTLLSQESLMASSVSAPCNEKLIQDQFVDISFPS
     . ... ......*. . ..... ...**  . ....*....   ...* ..  . .*...
256" PSRKMVENNLVVETGSQEKQESAFSPNT-PCTGEFIATDLAMNDKDMLSENSSMCLSLQE

657' QVVNTNMQSVQLNTEDTVNTKSV--NNTDATGLIQGVKSVEIEKDAQLKQFLTPKTEQLK
        ..   **. . . . ...*.  ...* ..*..* ..  .....*..  *.* .*..
315" LNPASITSSVPKKHDPNPSDSSLAQSTGDRANLLNGEHG--LSSGQQINCELPP-VENI-

715' PERVTSQVSNLKKKETTADSQTTTSKSLQNQSLKENQKKPFVGSWVKGLISRGASFMP--
     . ..... .*... .. .... ..   *. ...*.. ******* *.*...**.*
371" -VQKSPDLTGASKTAVHSQVASSSVDNNSFQPSDKDQRRGFVGSWVKKLLSKNTSFLPSS

773' --LCVSAHNRNT-----ITDLQPSVKGVNNFGGFKTKGINQKASHVSKKARKSASKPPPI
       .  ......*     ..*   .***..*****...* ..*.*.. ...  ..... .
430" ASMPKNERSWKTPSVQKLSDTWLPVKGASNFGGFQGRG-EHKSSQTLTSVLPRSNNAQCL

826' SKPPAGPPSSNGTAAHPHAHAASEVLEKSGSTSCGAQINHSSYGNGISSANHEDLVEGQI
     **      * ..  ...*.  .. . .***.*  *.  .   .  . .*.. *.  ....
489" SK--FQGFSQGTCLPRSHSAKEGPTWNKSGNTQGGSGKTTQVHPPSCNSVKAEESDSDKT

886' HKLRLKLRKKLKAEKKKLAAL--MSSPQSRTVRS--ENLEQVPQDGSPNDCESIEDLLNE
     .****** ***.*.*****.*  ..  *..  ..   .... .*..*.**.* ....*.*
547" RKLRLKLLKKLNAKKKKLASLDRLAVEQAKRGKPVARDVSAPSQTESHNDSELLQSFLRE

942' LPYPIDIASESACTTVPGVSLYSSQTHEEILAELLSPTP-VSTELSENGEGDFRYLGMGD
     *.*.** *..*.  .* . .  * ....*********.. *...   ..*.. .*..* .
607" LQYHIDAADNSSEFSVNSGCT-SHNSNDEILAELLSPASTVASSEVPKSEDECMYMEMVN

1001' SHIPPPV-PSEFNDVSQNTHLXQDHNYCSPTKKNPCEVQPDSLTNNACVRTLNLESPMKT
      *  ..*. *.* ..**...  ..*..*..*.*.. .*  ..  ....  .. ..*** ..
 666" SSSAAPAEPAEKTSVSHAAVTSEDRSYYGPVKDSNYE--RTVSKSSV--KKTGFESPTRE

1060' DIFDEFFSSSALNALANDTLDLPHFDEYLFENY
      **....** ** ...*.* .*.***** ***
 722" DILEDLFSISAPTTMAGD-MDVPHFDETLFETW
```

FIG. 4. Deduced amino acid sequence of chick retinovin and its homology to a human 120.4 kD protein (GenBank Accession No. X59131). The 120.4 kD protein shows 76.8% homology with chick retinovin and maybe its human counterpart. Upper lines: human 120.4 kD protein (301–1092 amino acids); lower lines: chick retinovin (1–753 amino acids). Asterisks: identity; dots: positivity.

From the sequence of the 498 bp-long cDNA fragment, 2 gene-specific primers were designed for forward and reverse extension by 3′-RACE and 5′-RACE, respectively. Poly(A)+ sequence located at the extended end was obtained by amplification with a gene-specific primer from the oligo-dT-primed cDNA, which determined the 5′-3′ orientation of this initially isolated cDNA fragment. Further three rounds of 5′-RACE were done by PCR with gene-specific primers from the gene-specific primer-primed cDNA to isolate the whole coding region (Fig. 3).

The alignment of six DNA fragments obtained as above resulted in the sequence with the length of 4120 bp (GenBank Accession No. AF242552). The sequence contained an open reading frame encoding a putative protein with 753 amino acids. The first round of 5′-

RACE generated two DNA fragments with different length. The sequence confirmed that the one fragment contained a 511-bp insert in the other fragment (Fig. 3). The insert was located in the 3′ untranslated region and might be derived from alternative splicing. The protein was named "retinovin," based on its selective expression in the early ("novus" in Latin) stage of retinal development. Northern blot analysis with a cDNA fragment located within the coding region as a probe showed that the retinovin message at about 12 kb was also expressed in the 15-day embryonic brain, but not in the 7-day embryonic brain (Fig. 2). In GenBank search (dbest), five expressed sequence tags (ESTs) identical to a part of the sequence of retinovin were found. These ESTs (GenBank Accession Nos. AJ394402, AJ393282, AJ396022, AJ397821, and AJ396729) were isolated from chicken bursa and their function remained unknown.

By MOTIF sequence motif search (www.motif.genome.ad.jp) for matching with the amino acid sequence of retinovin, one homologous sequence, human hypothetical 120.4 kD protein (GenBank Accession No. X59131), was found. This gene was isolated from a lambda gt11 library of the human melanoma cell line, IGR3, and was assigned to chromosome 13 (8). The 120.4 kD protein with 1092 amino acids has a highly charged amino acid sequence, but its function remains unknown. The region (365–1090 amino acids) of the 120.4 kD protein shows 76.8% homology (31.4% identity and 45.4% positivity) with the region (20–751 amino acids) of retinovin (Fig. 4). The 120.4 kD protein might be a human counterpart of chick retinovin.

The amino acid sequence of rhodopsin-like G protein-coupled receptor (GPCR) superfamily signature was also found at four locations within retinovin by MOTIF search (Fig. 3). A hydrophilicity/hydrophobicity plot revealed a few clusters of hydrophobic amino acids, suggestive of membrane-spanning domains. However, the putative protein did not have seven membrane-spanning domains as GPCR.

At present, the function of retinovin as well as whether it is a membrane protein or not remains unknown. At the 7-day embryonic stage when retinovin is expressed, the retina is thickened with proliferation of cells, but is still before the division into neuronal cell lineage and glial cell lineage. Retinovin might play a role in the division into either cell lineage or triggering the development of a multilayered structure of neurons in the retina. Furthermore, retinovin might be used as a marker for multipotent stem cells in the retina.

In contrast with the retina, retinovin was expressed in the brain at the 15-day embryonic stage, but not at the 7-day embryonic stage. The reverse pattern of retinovin expression between the retina and the brain might be explained by the difference in time course of the development between the two tissues. Further chronological analysis and localization of the expression in the retina and the brain as well as in the other tissues would elucidate the function of retinovin.

REFERENCES

1. Godbout, R. (1993) Identification and characterization of transcripts present at elevated levels in the undifferentiated chick retina. *Exp. Eye Res.* **56,** 95–106.
2. Jensen, A. M., and Raff, M. C. (1997) Continuous observation of multipotential retinal progenitor cells in clonal density culture. *Dev. Biol.* **188,** 267–279.
3. Reh, T. A., and Levine, E. M. (1998) Multipotential stem cells and progenitors in the vertebrate retina. *J. Neurobiol.* **36,** 206–220.
4. Adler, R. (1993) Plasticity and differentiation of retinal precursor cells. *Int. Rev. Cytol.* **146,** 145–190.
5. Matsuo, T., Takabatake, M., and Matsuo, N. (1997) The effects of growth factors on multicellular spheroids formed by chick embryonic retinal cells. *Acta Med. Okayama* **51,** 251–260.
6. Diatchenko, L., Lau, Y. F. C., Campbell, A. P., Chenchik, A., Moqadam, F., Huang, B., Lukyanov, S., Lukyanov, K., Gurskaya, N., and Sverdlov, E. D. (1996) Suppression subtractive hybridization: A method for generating differentially regulated or tissue-specific cDNA probes and libraries. *Proc. Natl. Acad. Sci. USA* **93,** 6025–6030.
7. Sato, Y., Matsuo, T., and Ohtsuki, H. (1999) A novel gene (oculomedin) induced by mechanical stretching in human trabecular cells of the eye. *Biochem. Biophys. Res. Commun.* **259,** 349–351.
8. Wilton, A. N., Zehavi-Feferman, T., Fleming, J., Baker, E., Chen, L. Z., and Cooper, D. W. (1991) A unique intronless gene or gene segment on chromosome 13 specifying a highly charged amino acid sequence. *Cytogenet. Cell. Genet.* **58,** 1985.