Vol. 213, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 4, 1995 Pages 181-188

# ISOLATION OF CANDIDATE GENES FOR MACULAR DEGENERATION USING AN IMPROVED SOLID-PHASE SUBTRACTIVE CLONING TECHNIQUE

**Timothy J. Schoen, Krzyzstof Mazuruk, Gerald J. Chader and Ignacio R. Rodriguez***

Laboratory of Retinal Cell and Molecular Biology, National Eye Institute,
National Institutes of Health, Bethesda, MD 20892

Received June 23, 1995

**SUMMARY:** An improved solid-phase subtraction procedure was developed to generate a readily amplifiable library of short cDNA fragments highly enriched in the macula (target) versus the peripheral region (driver) of the monkey neural retina. The generated clones were sequenced and 63 were analyzed by northern blotting using total RNA from the monkey macula and peripheral retina. The results indicate that 32% are highly enriched in macula, 36% are below the limits of detection and 32% are not enriched. No clones were found which were enriched in the peripheral retina. Our technique is therefore successful in identifying novel cDNAs enriched in the macula area of the neural retina that may represent potential candidate genes for hereditary ocular diseases. It should thus be useful in other situations where subtle differences in expression between cell types or tissue areas need to be analyzed. 

We have previously published a solid-phase subtraction technique for the isolation of tissue-specific genes that is relatively rapid and selective (1). The present modification, although more complex than the original, maintains the same general advantages while allowing the amplification and cloning of the subtracted fragments. We have used this technique to isolate cDNAs highly expressed in the macula region of the monkey neural retina by subtracting from a similarly-sized region in the peripheral retina. In the primate retina, the macula is an area of approximately 6 mm in diameter which exhibits structural and functional properties that are somewhat different from those of the peripheral retina. In particular, the macula has a high percentage of cone photoreceptor neurons and is considered to be the region mainly responsible for color vision and visual acuity (2). In contrast, the periphery is composed of the same basic neuronal cell types but

*To whom correspondence should be addressed at National Eye Institute, National Institutes of Health, Bldg. 6 Rm. 304, Bethesda, MD 20892. FAX# 301-402-0750, E-mail ignacio@helix.nih.gov.

0006-291X/95 $12.00

has a preponderance of rod photoreceptor cells and, at least in the primate, is much less important for functional vision.

The aims of the present study were threefold. First, to devise a general method that would yield clones that represent differences in mRNA expression between relatively similar cell types. Secondly, to specifically identify cDNAs that exhibit an enriched expression in the macula versus the periphery of the retina. Thus, we hope to obtain a better understanding of the specialized genes which allow for normal visual function in the primate. Thirdly, to determine the chromosomal localization of these clones and identify those which may be candidate genes for hereditary diseases involving the macula, e.g. Best's disease (3), Stargardt's disease (4). In this way, we hope to acquire a database of partially-characterized, macula-enriched cDNA clones at known chromosomal locations which may serve as candidates not only for the well-characterized diseases with known chromosomal locations, but also for other more widespread but much less well-understood conditions such as the age-related macular degenerations (ARMD).

## MATERIALS AND METHODS

**Materials.** Eyes from rhesus monkeys (*Macaca mulatta,* 2-3 yrs old) were obtained within 1 hr of sacrifice through the courtesy of the Center for Biologics Research and Testing, U.S. Food and Drug Administration (Bethesda, MD). Animal studies were conducted in accord with the NIH Guidelines on the Care and Use of Animals in Research. A 5 mm diameter region encompassing the macula was punched out using a trephine (Roboz, Rockville, MD). A similar punch was also taken in the peripheral (nasal) retina, approximately 5 mm from the *ora serrata.* The retina was carefully dissected off the underlying retinal pigment epithelium (RPE)-choroid-sclera complex and immediately frozen on dry ice and stored at -70°C before processing. Macula and peripheral retina punches from 125 eyes were pooled for use in northern blots and cDNA synthesis.

**RNA extraction and northern blot analysis.** Total RNA was isolated from the monkey retina tissue using the RNAzol technique (Cinna/Biotecx Laboratories, Friendswood, Texas). Northern blots were prepared by running 5 μg of total RNA per lane as previously described (5). The blots were probed with $^{32}$P-labeled PCR products (6) generated from individual clones.

**Solid-phase cDNA synthesis.** The cDNA synthesis on Dynabeads Oligo $dT_{(25)}$ (Dynal Inc., Lake Success, NY) was performed as previously described (1,7). A 100 μL aliquot (0.5 mg) of the beads was used for the synthesis. The synthesis should be performed in a 0.5 mL PCR tube with a top insert (Sartsted, #72.733.050, Germany) to avoid using oil. After the cDNA synthesis, the beads were restored to their original volume in 1X TE, 50% glycerol. The cDNA should be tested by PCR using primers to the 5' end of a known cDNA such as GAPDH, actin, etc.

**Polymerase Chain Reaction (PCR).** The PCR amplifications were performed using a 2X PCR mix as previously described (7).

**Anchor primer A ligation.** The anchor primer ligation to the 3' end of the macula cDNA was performed as previously described (7,8). Essentially, a 20 μL reaction mixture containing the macula beads (target, initially 100 μL), 10 μL 2X ligation buffer + ATP (8), 1μL anchor primer A (100 ng, P-CACGAATTCACTATCGATTCTGGAACCTTCAGAGG-$NH_3$), 7 μL $H_2O$ and 2 μL RNA ligase (New England Biolabs, Beverly, MA) was prepared. The anchor primer A and the macula cDNA were allowed to ligate for 12-18 hrs at 25°C. The beads were then washed and resuspended in 100 μL of 1X TE.

**Labeling of amplimer 1.** The amplimer 1 oligonucleotide was labeled using [$^{32}$P]γATP (Amersham, 3000 Ci/mmol) and polynucleotide kinase (Promega, Madison WI) as described (5).

**Linear amplification of target cDNA**. The anchor A ligated macula-beads (target, 5 µL) were added to a 0.5 mL amplification tube and bound using a magnet holder. The supernatant liquid was removed and the cDNA was linearly amplified using a reaction mixture containing the target cDNA, 10 µL of 5' $^{32}$P-amplimer 1 (~50 ng, $^{32}$P-TCCAGAATCGATAGTGAATTCGTG), 15 µL $H_2O$ and 25 µL of 2X PCR mix for a total of 50 µL. The amplification was performed for 20 cylces with 30 sec. denaturing (94°C), 30 sec annealing (60°C) and 30 sec. extension (72°C). The single stranded cDNA (sscDNA) was purified using a Select D G-50 spin column (5Prime-3Prime, Inc., Boulder, CO), Speed-Vac dried and dissolved in 50 µL of 5X SSC 5 mM EDTA. The sscDNA contained a range of size fragments from 200 bp to 1000 bp in length.

**Subtraction.** The peripheral retina beads (driver, 100 µL, 0.5 mg) were washed in 10X SSC and the supernatant liquid removed. The linearly-amplified single-stranded cDNA generated from (5 µL times 20 cycles) target macula beads (in 5X SSC, 5 mM EDTA, above) was added to the peripheral retina beads (0.5 mg) and allowed to hybridize for 24 hrs at 55°C. The subtraction was monitored by taking aliquots every few hours and assaying for radioactivity. When the subtraction hybridization reached a plateau, the target cDNA was removed and added to a new or regenerated set of driver beads. This step was repeated until complete subtraction (in this case > 95% loss of radioactivity) was obtained. The driver beads were regenerated by washing once with 0.2N NaOH followed by three washes with 1X TE or 10X SSC.

**Ligation of anchor B to subtracted cDNA.** The above subtracted material was desalted by passage through a Select D G-50 spin column equilibrated in $H_2O$. The cDNA was dried using a Speed-Vac and resuspended in $H_2O$. The ligation of the anchor primer B was achieved by dissolving the subtracted $^{32}$P-sscDNA in 3 µL $H_2O$ and adding 1 µL Anchor primer B (100 ng, 5'P-CGATGTCAGAGCTTCATGGATAGTCGGATCCGGT-$NH_3$), 5 µL 2X ligation buffer + ATP and 1 µL RNA ligase. The ligation was allowed to proceed for 12-18 hours at 25°C.

**Amplification of subtracted products**. The subtracted products were amplified using amplimers 1 and 2. In general, the amplification required 1 µL of the subtraction mix (above), 1 µL amplimer 1 (100 ng, TCCAGAATCGATAGTGAATTCGTG), 1 µL amplimer 2 (100 ng, ACCGGATCCGACTATCCATGAAGC), 22 µL $H_2O$ and 25 µL 2X PCR mix. The amplification was performed for 30 cycles at 60°C annealing, 1 min extension.

**Cloning of subtracted fragments.** The amplified material was cloned by either direct TA cloning using the Promega's pGEM-T Vector System II kit (Promega, Madison, WI) or by digesting it with EcoRI and BamHI and force cloning the fragments into the same sites in pBlueScript (Stratagene). The subtracted material can also be directly labeled and used to screen cDNA or genomic libraries.

**Purification of PCR products.** The cloned PCR products were purified using Promega's Wizard PCR Preps kit or Select II spin columns (5Prime-3Prime, Boulder, CO).

**Automated fluorescent sequencing.** Fluorescent sequencing was performed in a Perkin-Elmer/Applied Biosystems (ABI) model 370A instrument. The sequencing was performed using ABI's PRISM Ready Reaction cycle sequencing kit following the manufacturer's protocol. The reactions were purified using Select-D G-50 columns.

**Chromosomal localization by PCR.** The human monochromosomal somatic cell hybrid panel 2 was purchased from the NIGMS Human Genetic Mutant Cell Repository at the Coriel Institute for Medical Research (Camden, NJ). PCR amplification was performed using 0.3 µg of genomic DNA from panel samples and oligonucleotides specific to matching human Expressed Sequence Tags, ESTs (9).

## RESULTS

**Solid-phase subtraction**. The solid-phase subtraction was performed as shown in Fig. 1. Single-stranded cDNA was synthesized on Dynabeads Oligo $dT_{(25)}$ from macula RNA (TARGET) and from peripheral retina (DRIVER) as previously described (1,7). The anchor primer A was ligated

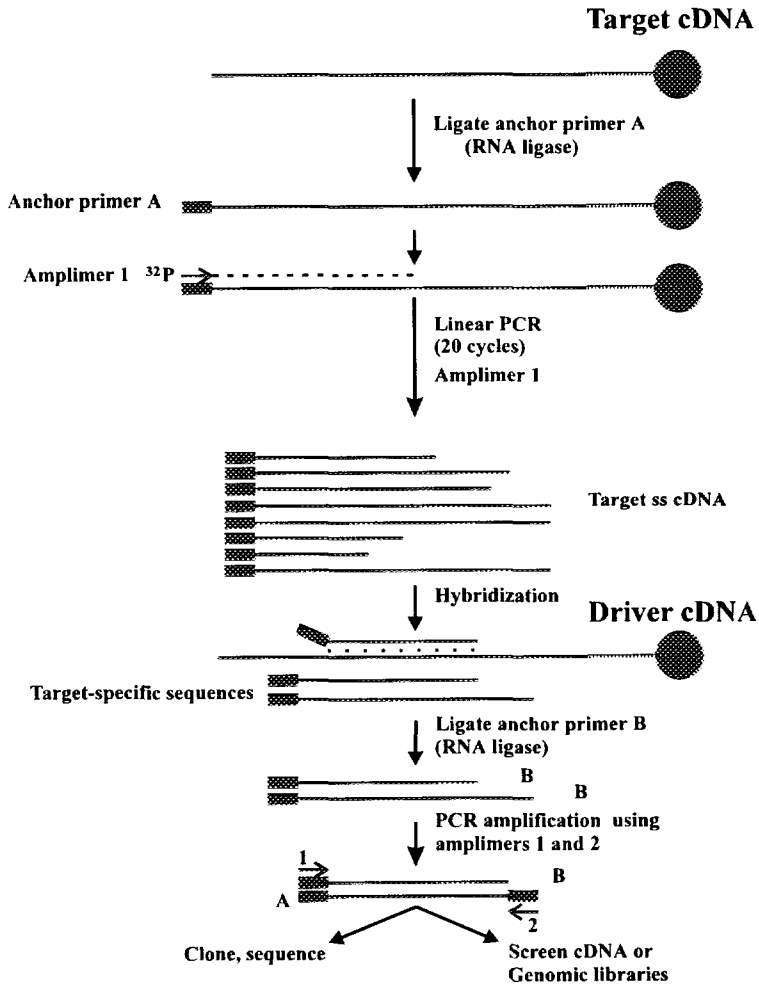


**Figure 1.** Schematic showing the solid-phase subtraction procedure.

to the 3' ends of the antisense macula sscDNA as previously described (7). A 5 μL aliquot of the macula-beads and a $^{32}$P-labeled oligonucleotide (amplimer 1 complementary to the anchor primer A) were used to linearly amplify the macula cDNA (TARGET). The $^{32}$P-labeled, sense, macula-derived sscDNA fragments were then added to 100 μL of the peripheral retina antisense sscDNA (DRIVER). The mixture was allowed to subtract for 18-24 hours. The unbound material was removed from the driver beads and then added to another 100 μL batch of peripheral retina beads. The subtraction process was repeated 3 times over 3 days. More than 95% of the original material was subtracted. The final unbound material was purified on a Select D G-50 spin column and dried. Anchor primer B was then ligated to the subtracted sscDNA and a small portion amplified using amplimers 1 and 2. The amplified products were digested with EcoRI and BamHI, purified and then force cloned in pBlueScript SK+ vector.

**Screening of the subtracted library**. The library was plated and each individual transformant was amplified directly from the bacterial colony using T3 and T7 vector-specific primers. The PCR products were purified and sequenced directly using the T3 or T7 primers. The sequences were edited and a GenBank search was performed using the BLAST server (10). Northern blots containing 5 μg of total RNA from macula and from peripheral retina were probed with the $^{32}$P-labeled PCR products derived from each individual isolate. The clones were organized into a database containing three main categories based on northern blot results. The main categories were: ENRICHED, UNDETECTABLE, and NOT ENRICHED (Table 1.). Each category contains three subcategories: KNOWN (match with a known sequence), UNKNOWN (no match) and EST (match to an Expressed Sequence Tag).

**Clone evaluation.** At this time, we have sequenced over 100 clones. Only 63 of these, however, have been subjected to northern blot analysis and the extent of macula enrichment determined. Representative northern blots can be seen in Fig. 2. Importantly, three of the clones obtained from

**Table 1.** List of clones sequenced and categorized by northern blot analysis and GenBank search

| EST | | UNKNOWN | KNOWN |
|---|---|---|---|
| **ENRICHED-32%** | | | |
| K2-Ch14 | K59-Pseudogenes | K34 | K63-Calmodulin |
| K32 | K61 | M6 | M1-Red Cone Pigment |
| K37-Pseudogenes | M4-Ch20 | M11 | M13-Cone Arrestin |
| K38-Ch1 | M5-Ch4 | M35 | M17-Proteosome Subunit HcC10-11 |
| K53 | M7-ChXq | | M22-Cone Arrestin |
| K57-Ch5 | | | |
| 11/63 (17.5%) | | 4/63 (6.3%) | 5/63 (8%) |
| **UNDETECTABLE-36%** | | | |
| K10 | M3-Pseudogenes | K54 | K11-Mel-18 protein |
| K12 | M16-Ch5 | M12 | |
| K24 | M19-Ch11p14-15.1 | M25 | |
| K56 | M23 | M33 | |
| K58 | M26-Ch2 | M34 | |
| K60 | M30-Ch7 | | |
| K74 | M31 | | |
| K79 | M32-Ch11p13 | | |
| M2 | | | |
| 17/63 (27%) | | 5/63 (8%) | 1/63 (1.6%) |
| **NOT ENICHED-32%** | | | |
| K7 | K28 | K4 | K1-U2 SnRNP auxillary factor |
| K13 | K35 | K39 | K46-Ubiquitin carrier protein |
| K14 | K43 | K48 | |
| K16 | K52 | M15 | |
| K18 | K66 | M20 | |
| K21 | K83 | M21 | |
| 12/63 (19%) | | 6/63 (9.5%) | 2/63 (3%) |

Where known, chromosomal assignment (Ch) is listed next to the clone number.

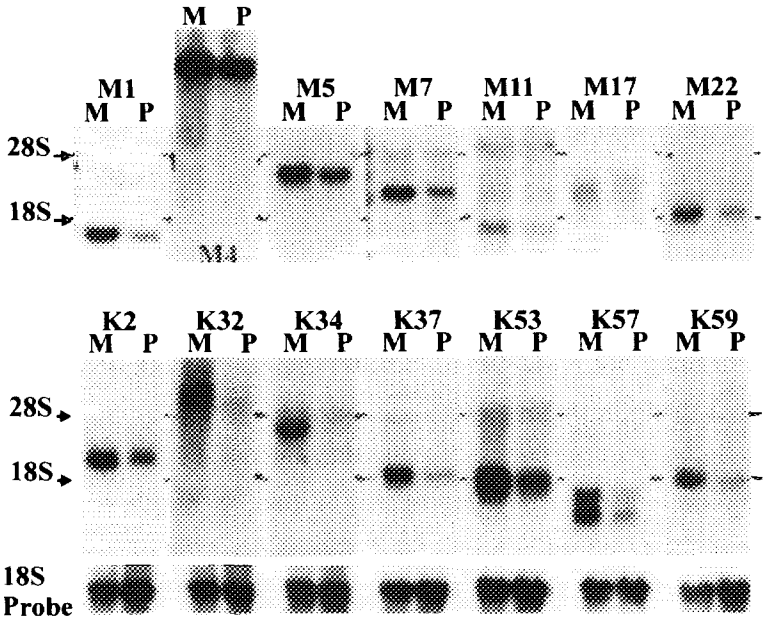


**Figure 2**. Northern blots containing RNA from the macula region (M) or peripheral region (P) of monkey retina. Each lane contains 5 μg or total RNA. The M (top row) and K (bottom row) designations for the clones signify two different screenings. The blots were aligned by positioning of 28S and 18S ribosomal RNA bands (shown by arrows); this also serves to estimate the sizes of the messages. Selected blots were probed with a fragment of human 18S RNA to determine relative loading levels.

the first screening were sequenced and found to be red cone pigment (M1) and cone arrestin (M13 and M22) as given in Fig. 2 and Table 1. These genes are known to be expressed only in cone photoreceptor neurons and therefore should be enriched in macula vs. peripheral retina. Several of our new isolates show macula enrichment as good or better than either the red cone pigment or cone arrestin (Fig. 2). Clones M7, M11, K32, K34, K57 and K59, for example, are highly enriched in the macular region. Of the macular-enriched clones, 11 of the sequences show 95-100% sequence homology to human ESTs while 4 are unique clones previously unreported (ENRICHED, Table 1). The mRNAs for a significant number of our clones are apparently in low abundance in the retina since they could not be detected by northern blot analysis (UNDETECTABLE, Table 1). Yet, these clones represent 16 other human ESTs and 5 additional potentially new genes. Even in the NOT ENRICHED category, 6 previously unreported sequences were obtained. Our technique thus not only gives a high percentage of macular-enriched clones and no peripheral-enriched clones but a large number of new sequences.

Many of our monkey cDNA clones matched (95-100% homology) with human ESTs in GenBank. Oligonucleotides were synthesized to the matching human ESTs and chromosomal

localization was performed by PCR on these selected clones. The chromosomal assignments for the apparent human cognates of several of the clones are listed in Table 1.

## DISCUSSION

We have used our solid-phase subtraction technique to isolate a number of cDNAs that are highly enriched in the macula versus the peripheral retina. Thanks to the recent influx of new Expressed Sequence Tags (ESTs) placed in GenBank (WashU-Merck EST Project), we have been able to easily convert many of our original monkey cDNA fragments into human sequences. As mentioned above, most of our monkey subtracted cDNA products have a 95-100% similarity to human ESTs. Knowing the human EST sequence has allowed us to pursue the chromosomal localization by PCR using NIGMS human-rodent somatic cell hybrids monochromosomal panels. We hope to follow our localizations by mapping each of our most promising cDNAs to an ordered YAC before proceeding with the cloning and characterization of the gene. This process should allow us to localize the genes of interest to particular microsatellite markers contained within the matching YACs and therefore to linked genetic diseases. Two of our clones M19 and M32 (Table 1.) map to two important areas in chromosome 11. M32 was localized to 11q13 where Best's disease is located (3) and M19 to 11q14-15.1 where Usher's syndrome type 1 is located (11). Ushers's is a syndrome with severe auditory and ocular manifestations.

Some of the most wide-spread and devastating diseases involving the macula are the age-related macular degenerations ARMD (12). This complex group of diseases demonstrate familial inheritance patterns suggesting genetic component(s) may be involved (13). The complex inheritance patterns, phenotypic variability and late onset of the disease, however, have greatly complicated linkage analysis studies. Recent technological advances, though, are making the linkage analysis of large numbers of patients more feasible. We hope this and the isolation of relatively large numbers of novel, macula-enriched cDNAs may prove helpful in elucidating the genes involved in ARMD.

Finally, the fact that the present technique yielded a relatively high percentage of retinal cDNAs that were highly enriched in the macula indicates the potential for similar success in other tissues. Region-specific genes of the brain, for example, may be able to be quickly and efficiently assessed. Similarly, the technique should be applicable to determining more subtle developmental, hormonal-induced as well as age-related changes in gene expression within a tissue or specific cell type. Finally, the ease of our technique coupled with established methods of PCR chromosomal localization should allow for the rapid accumulation of relatively large groupings of "candidate genes" of known tissue-specificity and chromosomal localization.

**ACKNOWLEDGMENT**

Dr. Mazuruk was supported as the John W. Kluge Research Fellow of the Foundation Fighting Blindness.

**REFERENCES**

1. Rodriguez, I.R. and Chader, G.J. (1992) *Nuc. Acids. Res.* 20, 3528.
2. Fine B. and Yanoff M. *Ocular Histology.* 2nd ed. San Francisco: Harper and Row (1979):111-122.
3. Weber, B.H.F., Walker, D., Muller, B. and Mar, L. (1994) *Genomics* 20, 267-274.
4. Kaplan, J., Gerber, S., Larget-Piet, D., Rozet, J.M, Dollfus, H., Dufier, J. L., Odent, S., Postel-Vinay, A., Janin. J., Briard, M.L., Frezal, J. and Munnich, A.(1993) *Nat. Genet.* 5, 308-311 Errata, (1994) *Nat. Genet.* 6:214. Zhang, K, Bither, P.P., Donoso, L.A., Seidman, J.G., Seidman, C.E., (1994) *Invest. Ophthalmol. Vis. Sci.* 35, 2143.
5. Sambrook J., Fritsch E.F., and Maniatis, T. (1989) "Molecular Cloning, a Laboratory Manual." 2nd edition. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, N.Y.
6. Showalter, D.B. and Sommer, S.S. (1989) *Anal. Biochem.* 177, 90-94.
7. Rodriguez, I. R., Mazuruk, K., Schoen, T.J., and Chader, G.J. (1994) *J. Biol. Chem.* 269, 31969-31977.
8. Tessier, D.C., Brousseau, R. and Vernet, T. (1986) *Anal. Biochem.* 158, 171-178.
9. Adams, M., Kelley, J., Gocayne, J., Dubnik, M., Polymeropolis, M, Xiao, H., Merrill, C., Wu, A., Olde, B., Moreno, R., McCombie, W. and Venter, J. *Science* 252, 1651-1656.
10. Altschul, S.F., Gish, W., Miller, W., Myers, E., and Lipman, D. (1990) *J. Mol. Biol.* 215, 403-410.
11. Smith, R.J.H, Lee, E.C., Kimberling, W.J., Daiger, S.P., Pelias, M.Z., Keats, B.J.B., Jay, M.L., Bird, A., Reardon, W., Guest, M., Agyagri, R. and Hejtmancik, F. (1992) *Genomics* 14, 995-1002.
12. Sarks, S.H., Sarks, J.P. (1989) Age-related macular degeneration: Atrophic. In: Retina, Ryan, S.J. (ed) (Medical Retina, vol 1.) St. Louis, MO, C.V. Mosby.
13. Silvestri, G., Johnston, P.B. and Hughes, A.E. (1994) *Eye* 8, 564-568.