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Topographical cone photopigment gene expression in deutan-type red–green color vision defects

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Abstract

Eye donors were identified who had X-chromosome photopigment gene arrays like those of living deuteranomalous men; the arrays contained two genes encoding long-wavelength sensitive (L) pigments as well as genes to encode middle-wavelength sensitive (M) photopigment. Ultrasensitive methods failed to detect the presence of M photopigment mRNA in the retinas of these deutan donors. This provides direct evidence that deuteranomaly is caused by the complete absence of M pigment mRNA. Additionally, for those eyes with mRNA corresponding to two different L-type photopigments, the ratio of mRNA from the first vs. downstream L genes was analyzed across the retinal topography. Results show that the pattern of first relative to downstream L gene expression in the deuteranomalous retina is similar to the pattern of L vs. M gene expression found in normal retinas.

Keywords: Color blindness; Gene expression; Photopigment genes; Deuteranomaly; Topography

1. Introduction

People with the common inherited color vision defects are missing a contribution from one class of cone photoreceptor. As there are three classes of cones, there are three classes of color vision defect. Those involving the short wavelength (S) photoreceptors are rare, while those involving the middle (M) or long (L) wavelength sensitive photoreceptors, collectively termed red–green color vision defects, are extraordinarily common. Deutan-type defects, which affect M cones, outnumber, by 3:1, protan-type defects which affect the L cones. Thus, the deutan-type color vision deficiencies, deuteranopia and deuteranomaly, are the most common forms of color blindness. In the United States, 1 out of 17 males and 1 out of 280 females have a deutan-type deficiency.

A known cause of deutan-type color vision defects, illustrated in Fig. 1A, is the loss of all genes encoding the M photopigments (Deeb et al., 1992; Nathans, Piantanida, Eddy, Shows, & Hogness, 1986). Deutan defects have also been found to be associated with deleterious point mutations in M photopigment genes (Bollinger, Bialozynski, Neitz, & Neitz, 2001; Ueyama et al., 2002; Winderickx et al., 1992). However, the above gene defects account for only a fraction of deutan disorders. For most people with deutan color vision defects, the appropriate photopigment genes are apparently intact (Deeb et al., 1992; Nathans, Piantanida, et al., 1986). Thus, the cause of the color vision defect has not been immediately apparent from examination of the genes.

The majority of visual pigment gene arrays underlying normal red–green color vision (Fig. 1B) have one L gene and one or more M genes (Drummond-Borg, Deeb, & Motulsky, 1989; Nathans, Thomas, & Hogness, 1986; Neitz, Neitz, & Grishok, 1995) with an L gene being in the most 5′ position (or first) in the array (Neitz, Neitz, & Kainz, 1996; Vollrath, Nathans, & Davis, 1988). In contrast, deutan men who have normal-appearing M genes (Fig. 1C) usually have two genes encoding pigments with maximum sensitivities in the long wavelength region of the spectrum (Nathans, Piantanida, et al., 1986). In these deutan arrays, the second L gene, also referred to as a 5′M 3′L hybrid gene, is placed between the typical L and M genes.

In the classical theory, deuteranomalous color vision was believed to be the result of mutations in the

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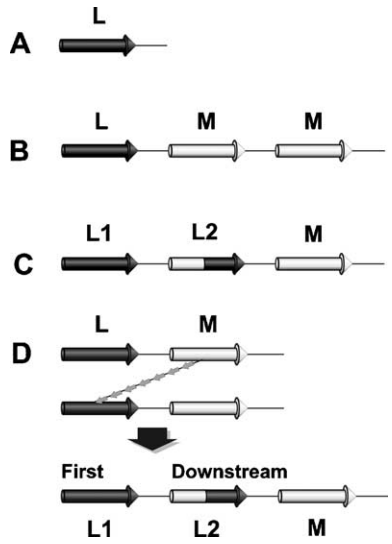

Fig. 1. Structure of the gene arrays from deutan or normal males. (A) A gene array that has lost all M genes will confer a deutan-type color vision defect on a male. In this case deletion of M genes leaves just one visual pigment gene on the X-chromosome. (B) A typical gene array from a color normal male has one L gene positioned first in the array followed by two M genes. (C) A typical array from a deuteranomalous male has two genes that encode L-class pigments, labeled L1 and L2, and a normal-appearing M gene. In the literature, the downstream L gene (L2) is referred to as a 5′M/3′L hybrid gene. (D) An unequal, homologous recombination between and M gene in one array and an L gene in another array produces a new array with the parental L gene (L1), a downstream L gene (L2), and the parental M gene, and is the mechanism believed to give rise to a typical deuteranomalous array.

M pigment gene which caused the encoded pigment to have an absorption spectrum that is shifted toward the long wavelengths. Within the context of this theory, the abnormal pigment was appropriately termed ''anomalous''. We now know that the classic theory is wrong, but the terminology associated with it has persisted. For example, the pigment encoded by the ''hybrid'' gene might be referred to as the ''anomalous'' pigment despite the fact that the coding sequence of the ''hybrid'' gene and the spectral sensitivity of the encoded ''anomalous'' pigment are not necessarily different from the large variety of L pigments encoded by the L genes that occur in the first position in the arrays from normal men (Carroll, McMahon, Neitz, & Neitz, 2000; Neitz et al., 1996), thus the terms ''hybrid'' and ''anomalous'' no longer seem appropriate. Here, in an attempt to avoid misunderstanding, we have replaced the term hybrid gene with ''*downstream L gene*'', referring to their two characteristic features: (1) relative to the first gene in the array (an L gene in normals) they are positioned downstream and (2) they encode a photopigment with a peak spectral sensitivity in the range of about 550–560 nm, qualifying them as encoding pigments of the L class (Asenjo, Rim, & Oprian, 1994; Merbs & Nathans, 1992; Neitz, Neitz, & Jacobs, 1989, 1991b).

Downstream L genes are not found exclusively in individuals with deutan color vision defects; they are also found in some individuals with normal color vision. (Drummond-Borg et al., 1989; Neitz et al., 1995; Sjoberg, Neitz, Balding, & Neitz, 1998). In addition, cadaver eyes have shown expression of multiple L genes as well as M genes. These results had led us to consider the possibility that the mutation that gives rise to the second L gene in deuteranomalous individuals might be independent of the mutation that caused the color vision defect. For example, in addition to the process that produced the downstream L gene, the M genes in deuteranomalous individuals might have specific mutations, that interrupt expression or function of the M pigment or viability of the M cones (Sjoberg et al., 1998). However, such mutations have only rarely been identified and thus alternative hypotheses have become attractive in which it is proposed that the addition of the second ''L'' gene and the loss of color vision are produced by a single mutational event.

Historically, two hypotheses have been proposed in which the addition of a downstream L gene is causative in deuteranomaly. An early theory, suggested by Nathans and colleagues (Nathans, Piantanida, et al., 1986; Nathans, Thomas, et al., 1986; Wang et al., 1999), assumed a model of transcriptional control of gene expression for the L and M pigments in which each normal M gene was proposed to have an M cone specific promoter at its 5′ end. Thus, an L gene displaced to a downstream position would be fused to an M gene promoter, theoretically leading to co-expression of the downstream L gene and the M gene in M cones. Nathans, Piantanida, et al. (1986) and Nathans, Thomas, et al. (1986) had speculated that such a misexpression of two pigments in the M cones could explain the characteristics of the color vision defect in deuteranomalous individuals. This was questioned based on psychophysical arguments (Neitz, Neitz, & Jacobs, 1991a) and recently evidence has accumulated for a stochastic model of gene expression (Smallwood, Wang, & Nathans, 2002; Wang et al., 1999), in which cone specific transcription factors are not required for the L and M pigments to be directed into separate cones. However, the co-expression theory has not been tested directly.

What has emerged as the most attractive theory is one proposed by Deeb and colleagues in which people with deuteranomaly lack or have very few M cones (Hayashi, Motulsky, & Deeb, 1999; Yamaguchi, Motulsky, & Deeb, 1997). In humans with normal color vision, L cones outnumber M cones by about 2:1 on average (Balding, Sjoberg, Neitz, & Neitz, 1998; Nerger & Cicerone, 1992; Pokorny, Smith, & Wesner, 1991; Roorda & Williams, 1999; Sjoberg et al., 1998). Deeb and colleagues have proposed that gene order determines whether a gene is expressed and that a gene positioned 3′ to the first two (or in the third position) is too distant

from upstream regulatory elements to be expressed. This hypothesis relies upon the well accepted idea, originally proposed by Nathans, Piantanida, et al. (1986) and Nathans, Thomas, et al. (1986), that the deuteranomalous genotype is produced by a single unequal intragenic crossover between two normal arrays. Since an L gene is normally first, when a three-gene array results from a crossover, the normal M gene is always displaced to the third position (Fig. 1D). To confirm this, Hayashi et al. (1999) determined the identity of the last gene in the array for a group of 10 deuteranomalous men whose arrays contained three genes, two L and one M. As expected, all had an M gene last. Next, Hayashi et al. (1999) took advantage of the fact that there are some men with normal color vision who have three genes, two of which encode L pigments. Their theory that the third gene is not usually transcribed predicts that in these individuals, a more complicated series of gene rearrangements must have occurred such that the downstream L gene is relocated to the third position. To test this they identified two color-normal male subjects who had two L and one M genes. Consistent with their hypothesis, both color-normal males had an L gene last in their arrays. In further support of the idea that M cones might be few or absent in deutan defects, Deeb et al. (1992) measured mRNA levels in cadaver eyes and found one example of an eye donor (color vision phenotype unknown) that had a gene arrangement similar to that of living deuteranomalous individuals and M pigment mRNA was not detected in his retina.

It is important to investigate this further by examining gene expression in additional eyes, and that is the purpose of the present experiments. Two considerations were taken into account in the experiments reported here. First, the experiment was designed to insure that cadaver eyes were from deuteranomalous individuals. Second we wanted to address the question of whether M gene expression, if it was found to be low in a given eye, was low enough to negatively impact color vision. The concerns come from the recent increased awareness that there is an enormous range among normal individuals in the ratio of L to M cones, and it is not unusual for people to have normal color vision with only a very small proportion of normal M (or L) cones (Carroll, Neitz, & Neitz, 2002; Carroll et al., 2000; Miyahara, Pokorny, Smith, Baron, & Baron, 1997; Roorda & Williams, 1999). Therefore, unless there is a method to specifically identify deutan eye donors, it is not possible be sure that an eye with low M expression is not from a color normal individual with an unusually small population of M cones. By systematically examining mRNA expression in a larger group of eyes, it is possible to answer the question of whether a reduction in the number of M cones is the most common cause of deuteranomaly and if it is, the accessory question can be answered of whether it is a reduction or complete absence of M cones that occurs in deutan defects.

In the experiments reported here, our approach to answering these questions was to start with the hypothesis that M genes are not expressed in deutan defects. We screened cadaver eyes from 150 males for the presence of M pigment mRNA. The frequency of deutan defects in the population is well established and the hypothesis that M genes are not expressed predicts that a number of eyes corresponding to the frequency of deutan defects in the population should lack M pigment mRNA. Nine such eyes were found in the sample of 150, corresponding exactly to the predicted 6% frequency of deutan defects in the population. In further experiments reported here, using two different ultrasensitive assays, the presence of M-pigment mRNA was undetectable in those nine eyes. This provides direct evidence that deutan defects, including those in which individuals have normal appearing M genes, are caused by the absence of M pigment mRNA.

The cadaver eyes identified here from men who had deutan color vision defects also provide a resource for testing hypotheses about the biological processes responsible for laying down the L and M cone mosaic. In arrays underlying normal color vision the first gene is L and the second gene is M. In deuteranomalous color vision defects both the first and second genes encode L pigments. The question is—what does this do to the architecture of the retina? In the normal retina, the first and second genes are each expressed exclusively in their own population of L and M cones (Hagstrom, Neitz, & Neitz, 2000). The processes responsible for directing the mutually exclusive expression of the two cone pigments into two respective cone populations are unknown and this is a major question that remains about the underlying biology of color vision.

Recently, a very simple hypothesis termed the ''stochastic model'' was proposed to explain how the exclusive expression of L and M pigments is directed into the cones (Wang et al., 1999). This model is an alternative to the one introduced above in which M and L cone specific promoter regions are supposed to interact with L and M cell-type specific regulatory proteins to control transcription of the photopigment genes. In contrast, in the stochastic model, the L and M cones are proposed to be molecularly indistinguishable until a random selector mechanism haphazardly chooses to express just one of the genes from the array in each cone. In doing so it produces the observed random mosaic of L and M cones in the normal retina (Roorda & Williams, 1999).

Although the L and M cones are randomly interspersed in the mosaic this does not imply that they are in equal numbers. As discussed above, in the central retina, on average, L cones outnumber M cones by 2:1. In addition, the ratio of L:M cones changes with retinal eccentricity so that the proportion of M cones is highest in the central retina and decreases in the periphery

(Hagstrom, Neitz, & Neitz, 1997, 1998). The stochastic model predicts that the pigment encoded by the downstream L gene would be topographically distributed in the retina in exactly the same pattern as the normal M cones. This is because, according to the model, the identity of the cone is entirely determined by the photopigment randomly chosen to be expressed within it. The rules that govern the probability that the gene in the second position is chosen for expression would be unaffected by whether the second gene encodes an M pigment, as in normal individuals, or an L pigment, as in deuteranomaly.

The distribution of "anomalous" cones in the deuteranomalous retina is a question that has long been of interest to color vision psychophysicists. In contrast to what would be predicted by the stochastic model, earlier results from psychophysical experiments had led to the suggestion that the distribution of the deuteranomalous pigment, in at least some deutan defects, might have nearly the opposite topographical expression pattern to that seen for normal M pigment, being low or absent in the central retina but increasing in proportion in more peripheral retina. This had been proposed to explain why some people with more severe deutan phenotypes tend to behave as dichromats when colored stimuli are small and centered near the fovea but behave as anomalous trichromats when stimuli are enlarged to extend into more peripheral retina (Nagy, 1980; Nagy & Boynton, 1979; Pokorny & Smith, 1977).

In the eyes of the deutan donors investigated here, the expression pattern of the first versus the second L gene was similar to the pattern observed for L versus M mRNA in the eyes of individuals with normal color vision. Thus, the large field trichromacy observed in some deuteranopes probably has an explanation other than an increase in the number of "anomalous" cones in the periphery. The similarities for first versus second gene expression in normal and deuteranomalous eyes independent of whether the second gene encodes an L or M pigment, are consistent with the stochastic model, as is the result that deuteranomals completely lack detectable M pigment mRNA. A finding of significant M gene expression would have presented a challenge to the stochastic model which predicts that each cone should express only one pigment gene from the array.

2. Methods

2.1. Screen for putative deutan retinas

Human eyes from 150 male donors were obtained through the Wisconsin Lions Eye Bank. Male donors ranged in age from 3 to 91 years, with an average age of 66, a median age of 71, and a modal age of 77. Eyes were enucleated and refrigerated within 5.5 h of death.

Retinas were removed and a 6 mm trephine was used to take punches of retina as illustrated in Fig. 2A. Nucleic acids were extracted from the retinal punches as previously described (Hagstrom et al., 2000).

The retina from donor no. 1194 was flat mounted onto a piece of nylon filter on which a 3.0 mm^2 grid had been printed (Fig. 2B). The retina was frozen and later cut into squares along the grid lines. Nucleic acids were extracted from each square. The final nucleic acid pellet from each grid square was resuspended in 50 μl of 10mM Tris, pH 8.0, 1 mM EDTA, pH 8.0.

An aliquot of retinal nucleic acid from each donor was used sequentially in a reverse transcriptase reaction (RT) and the polymerase chain reaction (PCR) to amplify an aliquot of L and M pigment cDNA containing exon 5. The reaction conditions and primers have been described elsewhere (Sjoberg et al., 1998). The forward

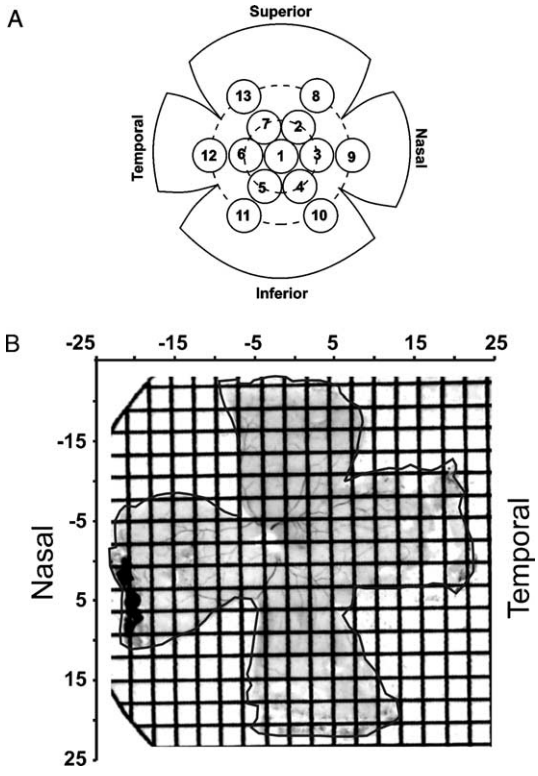

Fig. 2. Strategy for mapping the topography of X-chromosome visual pigment gene expression in human donor retinas. (A) Map of retina showing trephine punches used for analysis of L and M pigment gene expression. Punch 1 is centered on the fovea. Punches 2–7 were combined, and punches 8–13 were combined. Nucleic acid extracted from these three samples (punch 1, punches 2–7, and punches 8–13) were used in real-time quantitative PCR to analyze the relative expression levels of L and M photopigment genes (normal donors) or L1 and L2 photopigment genes (deuteranomalous donors). (B) Photograph of flat-mounted retina 1194 on a nylon filter printed with a 3 mm^2 grid. Retina was taken from the donor's right eye. Retinal eccentricity in millimeters is indicated on the horizontal and vertical axes. Nucleic acids extracted from individual grid squares were used in a reverse transcriptase reaction followed by real-time quantitative PCR to estimate the relative expression level of L1 and L2 photopigment genes.

PCR primer spans the exon 4–5 junction and thus will not amplify genomic DNA. The reverse primer corresponds to the 3′end of exon 5. The resulting PCR product was cleaved with restriction endonuclease *Rsa 1* which cleaves exon 5 of L pigment cDNA, but not M pigment cDNA. If a retina lacks M pigment mRNA, this assay will yield only the *Rsa 1* cleaved exon 5 PCR product, and there will be no full-length PCR product.

2.2. Determination of the ratio of L/M genes by real-time quantitative PCR

A Sequence Detection System 7700 (Applied Biosystems, Foster City, CA) was used to estimate the ratio of L/M genes and the ratio of first/downstream genes as previously described (Neitz & Neitz, 2001). Breifly, a real-time quantitative PCR assay, which utilizes TaqMan chemistry was used to determine the L/M gene ratio in genomic DNA from each subject, as well as the ratio of first/downstream genes. For both assays, the TaqMan kit was used as recommended by the manufacturer.

2.3. DNA sequence analysis

In order to identify nucleotide sequence differences between the first and downstream L genes in putative deutan donors we sequenced the L genes. A genomic DNA fragment was amplified specifically from the L genes. It extended from within intron 2 (near the 5′ end of exon 2) to the middle of exon 5. PCR primers and conditions were described previously (Sjoberg et al., 1998). The resulting PCR product was used to amplify and directly sequence exon 3 of the L genes using automated fluorescent sequencing with dye-primer chemistry (Sjoberg et al., 1998). In addition, long distance PCR was done to amplify the first gene in the array (Neitz et al., 1996). The long-distance PCR product was used as a template to amplify exon three for direct, dye primer sequencing. Primers and conditions were those described by Neitz et al. (1996) except that the primers were tagged with the M13 forward or reverse primer sequences for dye-primer sequencing.

2.4. Determination of the ratio of L1/L2 mRNA by real-time quantitative PCR

Real-time PCR was used to estimate the ratio of mRNA from the first (L1) versus downstream (L2) L gene. Reverse transcriptase and PCR reactions were carried out sequentially in the same tube. PCR was done using the TaqMan Kit (Applied Biosystems, Foster City, CA) following the manufacturers instructions. Each reaction contained a final concentration of 5 mM $MgCl_2$, 0.3 mM each dATP, dUTP, dCTP, and dGTP, 250 nM L1 probe, 300 nM L2 probe, 900 nM each of the forward and reverse primers, and 0.5 units MuLV reverse transcriptase, and 0.6 units Rnase inhibitor. The forward primer corresponded to sequences that span the junction between exons 2 and 3; its sequence was 5′CTGTGTGGGATCACAGGTCTCT. The reverse primer corresponded to sequences within exon 3 and its sequence was 5′CCTTTGGCAATGTGAGATTTGA. The primers sequences were present in both L1 and L2 genes. The probe that was identical to the L1 genes was labeled at the 5′ end with VIC; the probe that was identical to the L2 genes was labeled at the 5′ end with 6-FAM. Both probes were tagged with TAMARA at the 3′ end. The sequence of the L1-gene probe was 5′TCATTTCCTGGGAGAGGTGGCTGG. The sequence of the L2-gene probe was 5′CATTTCCTGGGAGAGATGGATGGTGG. Although the L1 and L2 probes differ in length, the sequences recognized by them differ with respect to one another at the positions underlined above in their sequences. Reactions were incubated at 48 °C for 30 min, then at 95 °C for 10 min to complete the reverse transcriptase reaction. They were then incubated at 97 °C for 30 s, 66 °C for 1 min for 3 cycles, followed by 37 cycles of 95 °C for 15 , and 66 °C for 1 min. Sufficient template was added to each reaction so that the fluorescence intensity from the reporter dyes exceeded background fluorescence between cycles 16 and 24. A standard curve was developed for the L1/L2 real-time quantitative mRNA assay using known ratios of cDNA clones that either contained the L1 probe sequences or the L2 probe sequence. The standard curve is shown in Fig. 3. For each sample analyzed, the L1:L2 ratio was calculated as the average of triplicate independent measurements.

2.5. Quantitative analysis of M gene expression in presumed deutan retinas using real-time quantitative PCR analysis and restriction digestion analysis

Nucleic acid was isolated from five presumably deutan retinas using a previously described method (Hagstrom et al., 2000). Quantitative, real-time RT–PCR was performed using probes specific for M and L cDNA as previously described (Balding et al., 1998). As an additional method of confirmation, RNA was reverse transcribed and restriction digestion analysis was performed. This was done by nonspecifically amplifying exon 4 and part of exon 5 of the L and M mRNA from three presumed deutan donors. The forward primer corresponded to sequences that span the junction between exons 3 and 4 and its sequence was 5′CATCTTTGGTTGGAGCAGGTACTGG. The reverse primer corresponded to sequences within exon 5 and its sequence was 5′GGGTTGGCAGCAGCAAAGCAT. The resulting PCR product was end-labeled with ^{32}P then cleaved with restriction endonuclease *DdeI*, which cuts at a restriction site within the M pigment cDNA but

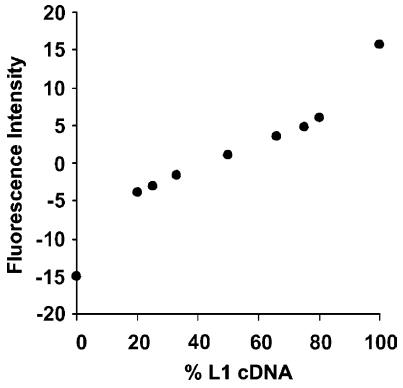

Fig. 3. Standard curve for real-time quantitative PCR measurements. Standards were created by mixing two cDNA clones, one containing the L1-specific probe sequence, the other containing the L2-specific probe sequence.

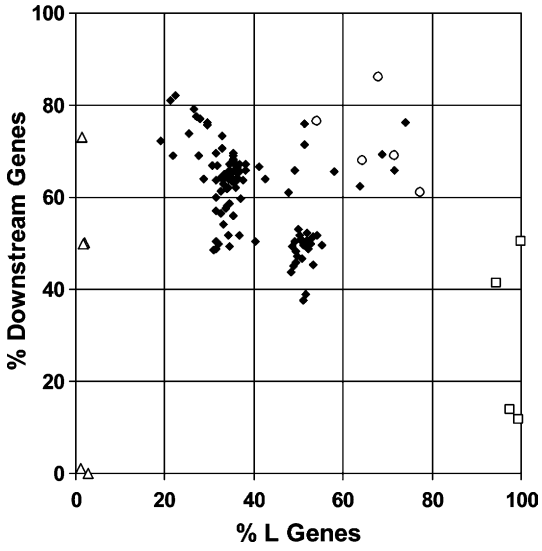

Fig. 4. Graphical representation of photopigment gene array structures for 150 male eye donors. Black diamonds are data from presumed color normal donors, white triangles are data from obligate protan donors (0% L genes), and white squares are data from obligate deutan donors (100% L genes). White circles are data from donors estimated to have two L genes and one or more M genes and are presumed deuteranomalous males. %L genes = 100 × L/(L + M); % downstream genes = 100 × downstream genes/(downstream genes + 1).

not within the L pigment cDNA. The resulting fragments were separated by electrophoresis in an 8% neutral polyacrylamide gel and visualized by phosphorimage analysis. The full-length PCR product was 323 base pairs (bp), whereas the products of the DdeI cleavage were 190 and 133 bp fragments.

3. Results

3.1. Identifying eye donors with deutan color vision defects

Initial non-quantitative analysis of L and M gene expression was done using retinal cDNA made by reverse transcription of mRNA in nucleic acid extracts from foveal punches from male eye donors. A segment of the L and M pigment cDNAs containing exon 5 was amplified from each donor and digested with *Rsa I*. There is an *Rsa I* restriction site in exon 5 of L pigment genes but not in M pigment genes. Donor retinas containing both L and M pigment mRNAs had cleavage products corresponding to uncut (M pigment cDNA) and cut (L pigment cDNA) PCR product, while those donors who lacked M pigment mRNA displayed only cut product. Out of a population of 150 male eye donors, we identified nine who lacked detectable M photopigment mRNA (open circles and open squares, Fig. 4).

For each of the 150 eye donors including the 9 who lacked detectable M gene expression, the number and ratio of L:M genes was determined using two real-time quantitative PCR assays (Sjoberg, unpublished results). One assay estimates the relative ratio of first to downstream genes and provides an estimate of the number of genes in each array. The second assay estimates the ratio of L:M genes (Neitz & Neitz, 2001). Fig. 4 is a graphical representation of the estimated photopigment gene array structures for each donor. The estimates for the number of downstream genes, expressed as a percentage of the total number of genes, are plotted on the *Y*-axis, and estimates for the number of L genes, expressed as a percentage of L + M genes are plotted on the *X*-axis. Donors whose arrays lack L genes (open triangles, Fig. 4) are obligate protans; donors whose arrays lack M genes (open squares, Fig. 4) are obligate deutans. Four of the nine donors who lacked detectable M pigment mRNA had arrays that did not have M genes (open squares, Fig. 4). The remaining five donors had M photopigment genes as well as two L genes (open circles, Fig. 4). These latter five donors were analyzed further with the goal of determining whether M pigment mRNA was completely absent.

3.2. M gene expression in deutan color vision defects

Two methods were used to quantify M pigment mRNA in the retinas of the five presumably deutan donors. First, quantitative, real-time PCR was performed on nucleic acid preparations from foveal punches, and the results are shown in Fig. 5. Quantitation of L and M pigment mRNA for the five putative deutan donors who had genomic M genes is shown in Fig. 5 (LM1, LM2, LM3, LM5, LM7). Using this method, we could verify that L pigment mRNA was present in the same sample that was assayed for M pigment mRNA. The PCR process is very efficient and until reagents become limiting the amount of product approximately doubles with every cycle. Fluorescence from the M-

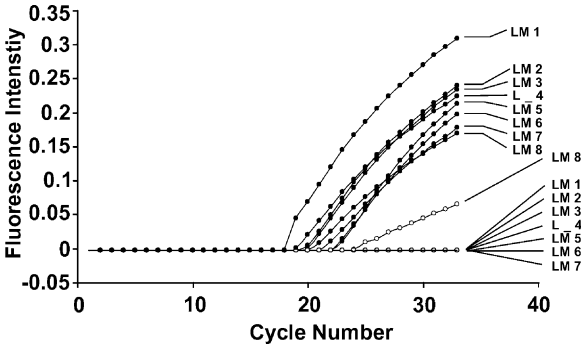

Fig. 5. Real-time quantitative RT–PCR amplification plots. Fluorescence intensities (arbitrary units) from the L-cDNA specific probes are indicated by closed circles; fluorescence intensities from the M-cDNA specific probes are indicated by open circles. Curves labeled LM1, LM2, LM3, LM5, LM6 and LM7 are from deutan retinas from donors with genomic M genes. LM4 is from a deutan retina, from a donor lacking genomic M genes. LM 8 is a control retina, previously estimated to have 10% M mRNA.

specific probe did not rise above background until after 37 PCR cycles, which was 15–16 cycles after the L-specific probe fluorescence exceeded background. Since the amount of PCR product is doubled with each cycle, this means that M pigment mRNA was present at a level of fewer than one copy of M in 2^{15} or 2^{16} copies of L or fewer than about one copy in 50 thousand. A normal donor (LM8) who had been previously characterized as having approximately 10% M pigment mRNA was used as a positive control and to demonstrate that a low level of M pigment mRNA, when present, is easily detected with this assay. In addition, a donor characterized as deutan based upon the absence of genomic M genes was used as a negative control (L_4).

Phosphorimage analysis of ^{32}P end-labeled cDNA was used as a second method to determine whether there was residual M pigment mRNA in three of the five presumed deutan donor retinas. Exon four was amplified from L and M pigment cDNA derived from reverse transcribed mRNA from two of the five donors previously described as well as from the center, mid-periphery, and far-periphery of the single retina acquired from a separate study (Fig. 2B). Amplified cDNA was first end-labeled with ^{32}P then digested with *DdeI*, which cuts at a restriction site within the M but not L cDNA. Digested and labeled PCR product was separated on an 8% polyacrylamide gel and visualized by phosphorimage analysis (Fig. 6). A normal retina containing approximately 10% M photopigment mRNA served as a positive control as described above (Fig. 5, LM8) and a deutan retina from a donor that lacked M genes was used again as a negative control. L and M cDNA clones were also used as controls to verify the efficiency of the digest. This technique also failed to detect M pigment mRNA in any of the presumed deutan retinas analyzed.

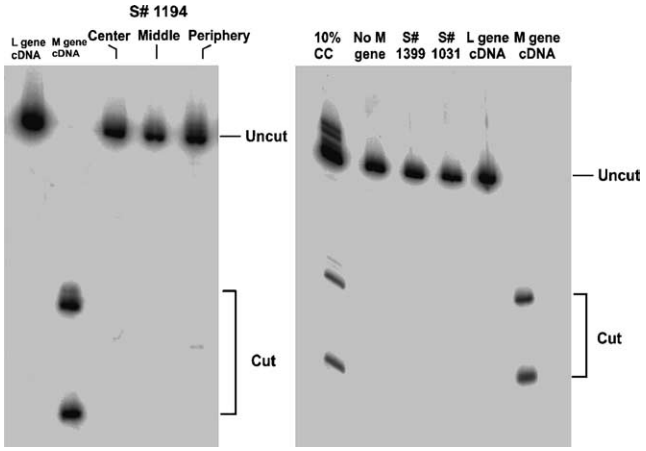

Fig. 6. Phosphorimage of P32 end-labeled cDNA, amplified from deutan retinas and digested with the restriction enzyme *DdeI*. Results from two of the five presumed deutan donors who had genomic M genes are shown on the right gel (S#1399 and S#1031), and results from the central (punch 1, Fig. 2), mid-peripheral (combined punches 2–7, Fig. 2) and peripheral retina (combined punches 8–13, Fig. 2) of the one additional donor, retina 1194, are shown on the left gel. An obligate deutan (no genomic M gene) was used as a negative control, and a color normal individual known to express 10% M mRNA and 90% L mRNA (10% CC) was used as a positive control. Cloned L and M pigment cDNAs were also used as positive controls.

3.3. Topographical analysis of gene expression in the three deutan retinas

In order to better understand the factors contributing to the absence of M pigment mRNA in these donors as well as gain information important for evaluation of the models put forth to explain the general mechanisms of photopigment gene expression, the relationship between gene position and expression was assessed for the first and the downstream L genes for three deutan donors. The donor whose retina was flat-mounted onto a nylon filter had two L and one M genes (Fig. 2B). Genomic sequencing revealed that his L genes differed by three nucleotide polymorphisms, a subset of which correspond to the difference between the L1 and L2 probes described in the methods. The retina was cut into squares and real-time quantitative PCR was used to estimate the ratio of L1:L2 mRNA in each square.

The measurements of first (L1) versus downstream (L2) L pigment mRNA were compared to the standard curve (Fig. 3) generated from known ratios of cloned photopigment cDNAs containing the L1 or L2 probe sequence. In order to estimate experimental error, the reproducibility and reliability of the assay was assessed by making repeated measurements using retinal samples from two male eye donors. A standard measurement from one of these two retinas was made along with every set of experimental measurements in order to insure that the system remained calibrated. These standard

measurements are shown in Fig. 7. In addition, the reliability of the experimental measurements over time was evaluated directly by measuring the ratio of L1:L2 mRNA in eight 3 mm^2 points along the horizontal meridian of the retina three times, in triplicate each time, over the course of four months. The results of these measurements are shown in Fig. 8. Triplicate measurements were made for each square across the retina. Expression ratios averaged from measurements in three adjacent squares along the horizontal and vertical axes (Fig. 3) are plotted in Fig. 9A and B.

There was more mRNA derived from the first gene than from the downstream L gene across the entire retina. However, first gene expression was highest at the periphery of the retina, composing, on average, about 85% of the L pigment mRNA, then decreased sequentially toward the center of the retina to compose 59% of the L mRNA at the fovea. Reciprocally, downstream L pigment mRNA was highest at the fovea and decreased outward toward the periphery. The topographical distribution of mRNA from the downstream L gene mimics that of M pigment mRNA in normal retinas. In addition, the gradient of mRNA from the first gene, that occurs across the deutan retina follows a very similar pattern to that observed for first pigment mRNA in normal individuals.

Two additional deutan retinas from the original population of 150 male eye donors had 2 L genes that differed by exon 3 polymorphisms, allowing us to quantify the ratio of L1 to L2 mRNA in these two retinas. Nucleic acid from circular punches of 6 mm diameter at the center, mid-periphery, and far periphery of the retinas was used in real time RT–PCR. Results, shown in Fig. 10, were consistent with the high resolution analysis described in Fig. 9.

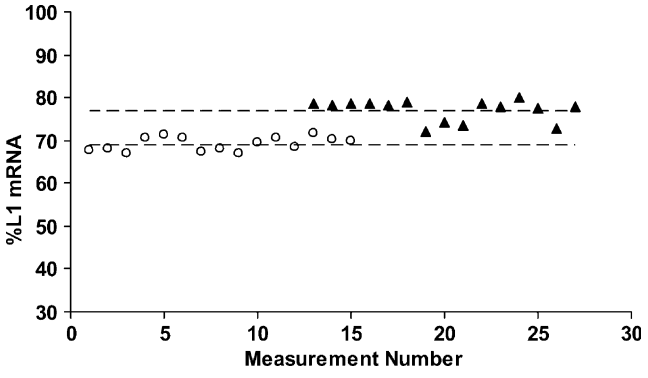

Fig. 7. Reliability measurement for real-time quantitative PCR. One of two retinal samples was used to standardize each set of quantitative measurements. Open circles indicate measurements made from the first standard retina; closed triangles represent measurements made from the second standard retina. Dashed lines represent the average for each set of measurements. Reactions were done in triplicate on 96-well plates so three standard measurements were taken per plate. No standard measurement differed by more than ±5% from the mean and 87% of standard measurements fell between ±2% of the mean.

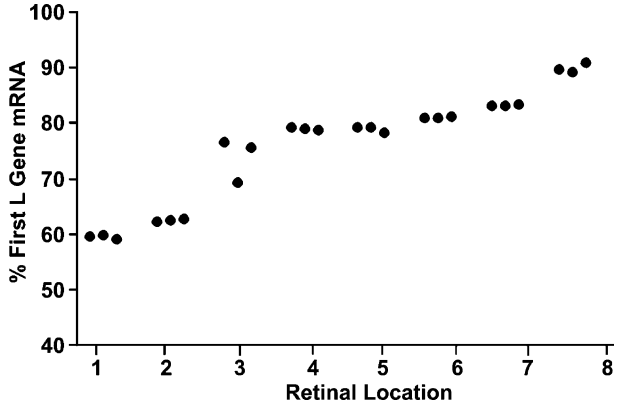

Fig. 8. Direct reliability measurements. Three triplicate sets of measurements were made for each of eight 3 mm squares across the horizontal axis of retina 1194. Averages of each of the three triplicate sets are plotted. No individual measurement fell outside ±5% of the set average and 96% of measurements fell within ±1%.

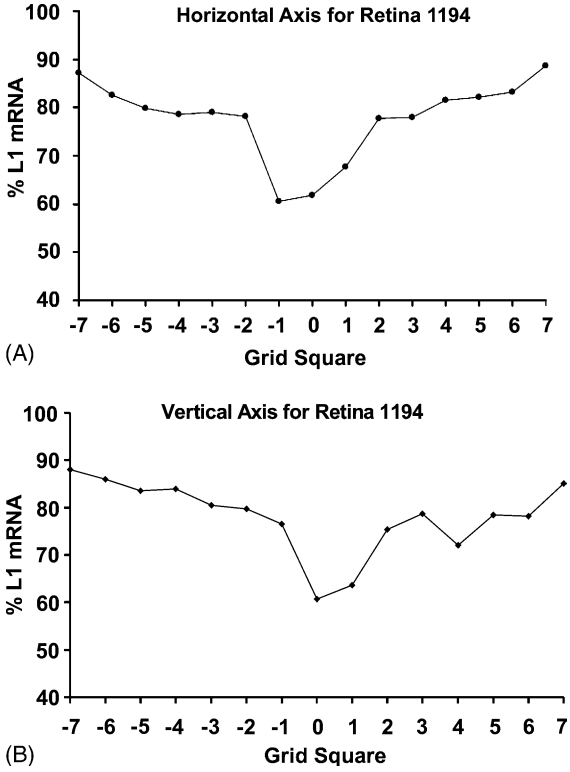

Fig. 9. Proportion of L1 pigment mRNA expressed as a percentage of L1 plus L2 mRNA plotted as a function of location on the horizontal (A) or vertical (B) axis. Each point on the horizontal axis plot represents the average of measurements made for vertically adjacent squares lying along the horizontal axis. Each point along the vertical axis represents the average of measurements made for three horizontally adjacent squares lying along the vertical axis. The fovea was in grid square 0. Negative numbers are nasal and inferior and positive numbers are temporal and superior.

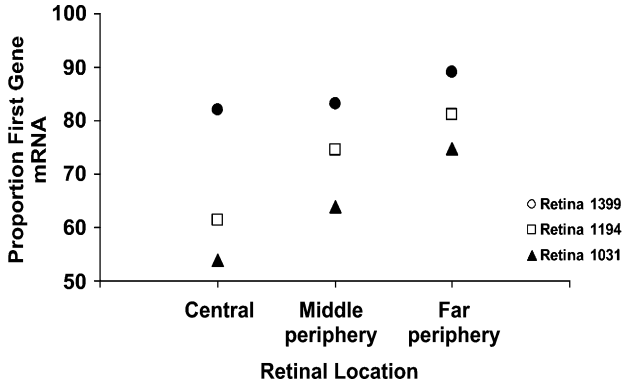

Fig. 10. L1:L2 mRNA for three deutan retinas measured as a function of eccentricity. The proportion of L1 mRNA was measured at central, mid-peripheral, and far-peripheral retinal locations. For all three retinas, % L1 mRNA was found to be lowest at the center of the retina and highest at the far-periphery.

4. Discussion

From the screening of 150 donor retinas nine were found to be lacking M pigment mRNA. This number (6%) exactly matches the frequency of deutan color vision deficiencies in the population as predicted by the hypothesis that the absence of M gene expression is the main cause of deutan color blindness. Five of these nine deutan retinas were from donors who had normal-appearing M photopigment genes as well as two genes encoding L-type pigments. No M photopigment mRNA was detected in tissue derived from the retinas of these deuteranomalous donors indicating that M pigment mRNA is completely absent in deuteranomalous eyes. In addition, examination of the topographical pattern of mRNA from first and downstream genes revealed that expression of the first (L) gene predominates and that relative expression of the downstream L gene decreases with retinal eccentricity. Thus, the pattern of first versus downstream gene expression across the retinal topography (Hagstrom et al., 1997, 1998) is similar regardless of whether the retina examined is deutan or normal or whether the downstream genes expressed encode L or M pigments.

Examination of photopigment mRNA provides results that can be used to distinguish among theories for the mechanisms that underlie deutan color blindness. The absence of M pigment mRNA from the five deutan retinas from donors who had genomic M genes rules out colocalization of a hybrid L plus an M pigment to the same set of cones as a cause of deutan color blindness. Rather, it appears that the downstream L gene product replaces the M photopigment with respect to its topographical distribution in the retinas of these color defective individuals. This is evidence against a conventional model in which transcriptional regulators are required to direct cell-type specific pigment gene expression into the L and M cones. These findings are consistent with the earlier results of Nathans and colleagues (Smallwood et al., 2002; Wang et al., 1999) in which transgenic mice harboring an artificial human X-chromosome visual pigment gene array were examined. The artificial array contained the minimal locus control region, followed by a reporter gene driven by the minimal L gene promoter, followed by another reporter gene driven by the minimal M gene promoter. Mice would not have the L and M specific transcriptional regulators hypothesized in the conventional model, yet in the transgenic mice a large fraction of the cones exhibited mutually exclusive expression of the two reporter genes.

Additionally, these results allow us to determine whether a reduction or a complete loss of M cones is responsible for the color vision defect. The inability to detect M pigment mRNA does not completely rule out the possibility that it could be present at an exceedingly low level. However, we note that the human fovea contains about 100,000 cones. The sensitivity of these experiments (1 in 50,000) is equivalent to being able to detect the presence of just two M cones in the fovea if all other cones were L. From this it seems very likely that M cones are completely absent from the eyes of deuteranomalous men.

The results of the topographical analysis are also relevant to understanding how the cone photopigments are assigned to specific cone cell populations within the retina. The finding that the topographical expression pattern of the first versus downstream genes is similar for normal and deutan color vision is consistent with a stochastic model for photopigment gene expression (Wang et al., 1999). The exact details have yet to be worked out but the essence of the stochastic model is that factors that determine which gene will be expressed are inherent to features of the gene array and its cis-acting regulatory elements. We assume that each individual cone has about the same amount of photopigment mRNA and thus the mRNA ratio measured in the experiments reported here reflects the cone ratio (Hagstrom et al., 2000). Accordingly, the finding that the first gene is expressed at a higher level in both normal and deutan retinas is consistent with the cones' identity being determined by elements inherent to the array and there being a relationship between the probability that a given gene is chosen for expression and its proximity to the 5′ end of the array.

The fundamental idea of the stochastic model is that the identity of a cone as being L or M is determined by the random choice of which pigment gene from the array is expressed. This general model can be separated from any specific theory about the details of the selector mechanism. However, a specific theory has been proposed. It has been well established that an enhancer element 3.6 kb upstream of the array, termed the locus control region (LCR), is essential for gene expression. According to this theory (Hayashi et al., 1999), the LCR

itself acts as the random selector by forming a stable complex with the promoter region of just one of the genes in each cell. In this model the probability of expression of the different genes in the array is determined by the physical distance from the LCR (Hayashi et al., 1999). However, two results reported here are not necessarily consistent with the details of the specific stochastic model that has been proposed. First, since the physical distance between a gene and the LCR is the same for all cells, one would not expect the probability of expression to change with retinal eccentricity. If we do not invoke a higher rate of cell-death for one cone type in the peripheral retina, then the control of the probability that a gene is expressed must be more complicated than a simple dependence on the unchanging distance between the gene and upstream regulatory elements. Second, a simple distance model would not necessarily predict that the expression of the third gene in the array would be completely absent as observed here. The ratio L to M cones in normal individuals is variable (Carroll et al., 2000) and it is not unusual for the ratio to be near 1:1. Given such a small and variable change in the probability of expression from the first to the second gene, it would seem surprising that the difference in expression between the second and third gene would be so dramatic. Current theories do not explain these observations very well, highlighting the fact that our present understanding of the mechanisms that control expression of the X-chromosome pigment genes is incomplete.

Apart from the issues concerning the details of the specific mechanism, the results presented here add to the accumulated evidence in favor of a stochastic process for determining photopigment gene expression. This has significant implications for models of how the neural circuitry for red–green color vision arises. In more classic models, the L and M cones have been conceived of as having separate identities in addition to which photopigment genes they express; under such models, it has been possible to propose that the cones carry distinguishing labels, either molecules at the cell-surface or secreted at the synapse that allow them to be individually recognized by second order neurons (Calkins & Sterling, 1999). In the stochastic model, the cones only differ in the opsins they randomly choose to express. When we consider normal color vision, it remains as a possibility under the stochastic model that the opsins themselves serve as the labels. Mollon (1999) has proposed an analogy with the olfactory system in which the receptor cells must also make specific connections with second order neurons. In that system the odorant receptor molecules are expressed both in the cilia and at the nerve terminal. Amino acids in an extracellular loop, which could act as labels on the cell surface, are correlated with amino acids thought to determine odorant specificity. In the case of normal color vision, there are consistent amino acid differences between L and M opsins that occur in an extracellular loop or near the membrane surface at the boundary between a helix and a loop. Could these be labels that are used by second order neurons to recognize the L and M cones and establish the connections required for color vision? The fact that deuteranomalous trichromats are able to base color vision on the expression of only two L pigments would seem to rule this out as a possibility. The two pigments expressed are often extremely similar in amino acid sequence and any candidate amino acids in or near extracellular loops that exist between normal L and M pigments are not consistently present in the two L pigments of deuteranomaly (Neitz et al., 1996). Nonetheless, even though their color vision is not like normal, deuteranomalous men can have reasonably good trichromatic color vision indicating that their nervous system is able to correctly wire two different L-cone-subtypes, so that the spectral difference between them can be used for color vision.

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