Evaluating the human X-chromosome pigment gene promoter sequences as predictors of L:M cone ratio variation

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Men with normal color vision vary widely in the ratio of long- (L) to middle-wavelength sensitive (M) cones. This variation provides opportunities to test models for the mechanism that produces L versus M cones during development. The L and M photopigment genes lie in a tandem array. Each gene has a promoter, and upstream of each array there is a genetic element, termed the locus control region (LCR), that is required for the expression of both L and M pigment genes. During development, for each cell that has been determined to be an L or M cone, it has been proposed that the LCR acts as a stochastic selector which chooses one gene from the array to be expressed. In this model, the L and M promoters compete for contact with the LCR in each photoreceptor. Theoretically, the promoter that, by chance, is the first to successfully form a stable and permanent complex with the LCR commits the cell to a lifetime of exclusive expression of its associated gene. Under this model, it has been suggested that nucleotide differences in the promoters influence their ability to compete in forming a complex with the LCR. Thus, normal variation in L:M cone ratio is predicted to be associated with nucleotide polymorphisms in the promoters. Here we tested this hypothesis by comparing the L and M promoter sequences for 73 males with normal color vision for whom L:M cone ratio estimates had been obtained previously. The M gene promoter sequences were found to be identical for all 73 males and the L gene promoters were identical for 71 out of the 73 males. Two males had mutations where in each case the L promoter differed by one nucleotide substitution compared to normal. Both of the males with promoter mutations had unusual cone ratios which is consistent with the growing body of evidence indicating that the relative ability of the promoters to form a complex with the LCR is a factor in determining cone ratio. However, the vast majority of cone ratio differences were not associated with any difference in the promoter sequence. To explain the high degree of cone ratio variation among normal males, the mechanism that determines whether a cone is L or M must involve genetic elements that have a high degree of genetic polymorphism in the normal population. The results presented here indicate that there are additional genetic components of the mechanism which remain to be identified and incorporated into the present hypotheses.

Keywords: color vision, L:M cone ratio, cone photopigments, opsin genes

Introduction

It has been estimated that, on average, long-wavelength sensitive (L) cones outnumber middle-wavelength sensitive (M) cones in the central retina (Cicerone & Nerger, 1989; Dartnall et al., 1983; Hagstrom et al., 1998). Recently, adaptive optics imaging of the living human retina in two males with normal color vision showed one male to have an L:M cone ratio of about 1:1, while the other had a ratio of nearly 4:1. This study provided direct evidence that there is dramatic variation in the L:M ratio across humans (Roorda & Williams, 1999). More recently, the range and distribution of L:M cone ratios were investigated in a study of 62 Caucasian males with normal color vision using the flicker-photometric electroretinogram (FP-ERG) and individualized cone spectra deduced from the L pigment gene sequence for each subject (Carroll et al., 2002). Among the 62 men, the estimated L:M cone ratio varied from 1:3 to 13:1. A theory for the mechanism proposed to regulate L and M photopigment gene expression suggests the locus of the genetic polymorphism responsible for the cone ratio variation.

In Old World primates, including humans, the L and M cone photopigment genes lie in a head to tail tandem array on the X-chromosome. This arrangement arose via a gene duplication event that is proposed to occur after the split between Old and New World primates 40-60 million years ago; Old World primates have a tandem array, but most New World monkeys do not. The single X-chromosome visual pigment gene in New World monkeys is polymorphic, with multiple alleles encoding different spectral variants. Heterozygous females receive different

alleles on their two X-chromosomes and as a consequence of X-inactivation, expression of different alleles is segregated into separate populations of cones. Mutually exclusive expression is a prerequisite for harnessing the photopigments for trichromatic color vision, and indeed heterozygous New World monkey females behave as trichromats (Jacobs & Neitz, 1985, Jacobs & Neitz, 1987). In New World primates the choice to become an L or an M cone is based simply on the stochastic choice made in each cell of which X-chromosome to inactivate.
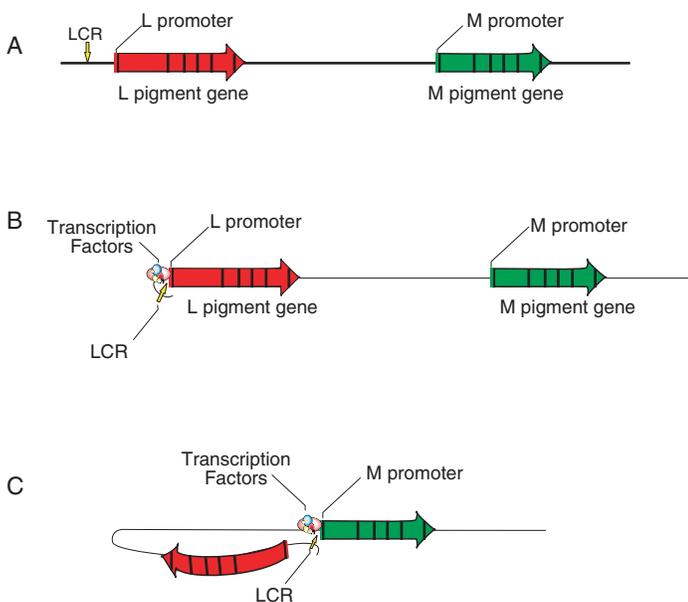

Figure 1. Model for mutually exclusive expression of L and M pigment genes proposed by Smallwood et al. (Smallwood et al., 2002). (A) Gene array in Old World primates showing duplicated region, and relative positions of the LCR and the L and M gene promoters. (B) The LCR is proposed to function as a stochastic selector that chooses whether a cell will express an L photopigment gene or (C) an M photopigment gene by forming a stable complex with L or M gene promoter that ensures exclusive expression of a single gene from the array in each photoreceptor.

Having L and M photopigment genes on the same X-chromosome in Old World primates requires a mechanism other than X-inactivation to segregate the expression of L and M genes into separate populations of cones. Nathans and colleagues have proposed a model for such a mechanism (Smallwood et al., 2002; Wang et al., 1999), which is illustrated in Figure 1. The sequences of the L and M pigment genes and the surrounding region of the X-chromosome indicate that the gene duplication extended from 236 base pairs (bp) 5' of the translational start codon to 24.4 kilobase pairs (kb) 3' of the translational stop codon. This implies that all of the necessary regulatory DNA elements necessary for cone-specific expression are contained within the duplicated region, and there is evi-

dence that the promoters for the L and M genes are contained within the 236 base pairs upstream of the translational start codons (Nathans et al., 1986; Shaaban & Deeb, 1998). A region of DNA that lies between 3.1 and 3.7 kb upstream of the transcriptional start site of the first (5'-most) gene in the X-chromosome array is an enhancer that plays an essential role in L and M gene transcription; deleting it from an otherwise intact array prevents expression of all X-chromosome photopigment genes and is a cause of blue cone monochromacy, a vision disorder characterized by the absence of functional L and M cones (Nathans et al., 1989; Nathans et al., 1993). In Old World primates, the enhancer has been termed the locus control region (LCR) and it has been proposed to function as the stochastic selector that chooses which one of the photopigment genes from the X-chromosome array will be expressed in an individual photoreceptor (Smallwood et al., 2002; Wang et al., 1992; Wang et al., 1999). Under this model the promoters for the L and M genes compete for contact with the LCR, and successful competition is envisioned to result in the formation of a stable and permanent complex between the LCR and the promoter of a randomly chosen gene in the array. Presumably, this process occurs in every cell destined to become an L or M photoreceptor, and the outcome of the competition commits the cell to the exclusive expression of a single X-chromosome photopigment gene for its lifetime.

Evidence from psychophysical measurements of L:M cone ratio among families suggested that variation in L:M cone ratio is an X-linked trait (DeVries 1948; Adam 1969). More recently, Smallwood et al. (Smallwood et al., 2002) proposed that nucleotide sequence variation in the L and M gene promoters might provide a tuning mechanism for the effectiveness of individual promoters in competing for contact with the LCR and forming a complex, and that such differences might be responsible for variation in the L:M cone ratio among men with normal color vision. The experiments reported here were designed to test this hypothesis, which predicts the existence of sequence variation in the L and M gene promoters would be associated with variation in cone ratio. We sequenced the L and M gene promoters for 73 males for whom the L:M cone ratio was previously estimated either using the FP-ERG, or from measurement of the L:M pigment mRNA ratio (Carroll et al., 2002). Despite the fact that the L:M ratios in this group of male subjects varied widely, there was very little nucleotide sequence diversity in the 236 bp segment of DNA upstream of each gene that contains the promoter. To explain the high degree of cone ratio variation among normal males, the mechanism that determines whether a cone is L or M must involve genetic elements that have a high degree of genetic polymorphism in the normal population. The results presented here demonstrate that there are additional genetic components of the mechanism which remain to be identified.

Primer pair	Name	Location*	Gene**	Primer sequence	concentration (nM)
1	FirstFwd	-418 to -394	L	5'CCTGGGCTTTCAAGAGAACCACATG	300
	LMx1Rev	51 to 71	L/M	5'TGGGTGCTGTCCTCATAGCTG	300
2	M2Fwd	-691 to -668	M	5'CCTGCAAGTGGGAATCTAAACAGA	300
	LMx1Rev	51 to 71	L/M	5'TGGGTGCTGTCCTCATAGCTG	100
3	MW+	-822 to -802	M	5'AGGTGATCCGGGAATAGTAAG	100
	LMx1Rev	51 to 71	L/M	5'TGGGTGCTGTCCTCATAGCTG	100
4	M1Fwd	-351 to -333	M	5'AGGCGTGTGCCACTGTGCC	100
	LMx1Rev	51 to 71	L/M	5'TGGGTGCTGTCCTCATAGCTG	100

Table 1. PCR primers. * Numbering system is the same as in Figure 2B, where +1 is the transcriptional start site. **Primers sequences occurs in L gene only (L), M gene only (M), or both L and M genes (L/M).

Methods

Subjects

The subjects were 7 living males for whom the L:M cone ratios were previously estimated using the FP-ERG and individualized L cone spectra predicted from the L pigment gene sequence for each subject, and 66 male eye donors for whom the L:M cone ratios were previously estimated from the ratio of the L:M photopigment mRNA in a 6 mm patch of retina centered on the fovea (Carroll et al., 2002). In a previous study, Hagstrom et al. (Hagstrom, Neitz et al. 2000) demonstrated that the L:M mRNA ratio provides a very good estimate of the L:M cone ratio by comparing the mRNA ratio to the ratios of cones obtained by direct counting of L and M cones in the same human donor retinas.

The performance of all living subjects on standard color vision tests indicated that they had normal color vision. The male eye donors were demonstrated to express both L and M pigment mRNA in the retina, and were thus presumed to be free of an inherited red-green color vision deficiency (Sjoberg et al., 1998).

Analysis of the L and M gene promoters

Genomic DNA was extracted from whole blood obtained from each subject as previously described (Poncz et al., 1983). An aliquot (1 nanogram) of DNA was used in the polymerase chain reaction (PCR). Hot start PCR was done using the XL PCR kit with AmpliWax Gem-100s. Each reaction contained 100 M each of dATP, dCTP, dGTP, and dTTP, and 1.4 mM magnesium acetate. Table 1 lists the PCR primer sequences, locations, and their final concentration in each reaction. Reactions with primer pair 1 were incubated at 95°C for 5 minutes, followed by 40 cycles of 94°C for 1 minute, 63°C for 1 minute, 72°C for 1 minute. Reactions with primer pair 2 or 4 were incubated at 95°C for 5 minutes followed by 40 cycles of 94°C for 1 minute, 60°C for 1 minute, 72°C for 1.5 minutes. Reactions with primer pair 3 were incubated at 95°C for 5 minutes, followed by 40 cycles of 94°C for 1 minute, 57°C for 1 minute, 72°C for 1.5 minutes. After completing 40 cycles

of the above described thermal cycling conditions, reactions were incubated at 72°C for 10 minutes.

A 489 bp fragment containing the L gene promoter was amplified with primer pair 1. Three separate fragments containing the M gene promoter were amplified. They were a 762 bp fragment using primer pair 2, a 893 bp fragment using primer pair 3, and a 422 bp fragment using primer pair 4. Primer MW+ was originally described by Dulai et al. (Dulai et al., 1999).

DNA sequence analysis

Automated fluorescent sequencing was done using AmpliTaq FS, and sequencing reactions were analyzed on an ABI Prism 310 Genetic Analyzer. Both strands of each PCR product were directly sequenced using the same primers used for PCR. Fragments containing the M gene promoter were also sequenced with the primer M3Fwd (5'CCAGCAAATCCCTCTGAGC) which hybridizes to a region of the M gene promoter 190 bp 5' to the transcription start site.

Results

Subjects for this study were chosen to represent the full range of variation in L:M cone ratio among men with normal color vision. Figure 2A shows a frequency histogram of the L:M cone ratios expressed as the percentage of L cones for each subject in this study.

The DNA sequences of the 236 bp containing the promoters were compared for L genes and for M genes for the subjects represented in Figure 2A. The results of the sequence comparisons are summarized in Figure 2B. All subjects shared the identical DNA sequence for the 236 bp upstream of the coding region for the M genes. All but two subjects shared the identical DNA sequence for the 236 bp upstream of the L genes. The two exceptions did not differ from other L gene promoters by random nucleotide substitutions. Instead, the L gene promoters had the nucleotides normally present in the M gene promoter at positions marked by asterisks in Figure 2B. The L gene promoter for one eye donor male who was estimated to have 50% L cones differed from the other L gene promoters by having

Figure 2. (A) Distribution of L:M cone ratios estimated by FP-ERG (dark bars, n = 7) or by mRNA analysis (light bars, n = 66). (B) Comparison of the DNA sequences for the 236 bp fragment containing the L and M gene promoters for the 73 subjects in (A). Asterisks indicate polymorphic nucleotide positions identified in L gene promoters from two subjects. Black asterisk indicates the single polymorphism in one subject; gray asterisks indicate 3 polymorphisms in a second subject.

the nucleotides normally found in M gene promoters at three positions, -166, -165 and -163. Another male who was estimated to have 92% L cones by FP-ERG analysis had a C (usually found in M gene promoters) at position -3 in his L gene promoter.

Discussion & conclusions

If, as hypothesized by Smallwood et al. (Smallwood et al., 2002), differentially tuning the competitive advantage of the L and M gene promoters in contacting and forming a stable complex with the LCR is the mechanism by which variations in the L:M cone ratio in human retinas are generated, then one would predict a high degree of sequence polymorphism in the L and M gene promoters associated with the variation in L:M cone ratio. Here, the 236 bp promoter regions upstream of the L and M photopigment genes were sequenced in a sample of 73 males who represent the entire range of L:M cone ratios observed in men with normal color vision. All 73 males had identical M gene promoters, and all but two males had identical L gene promoters. The two males that differed from the others in the L gene promoter sequences represent near extremes in L:M cone ratio (Figure 2A) with one male estimated to have 50% L cones and the other estimated to have 92% L cones. These nucleotide differences may contribute to the extreme L:M cone ratios for these two subjects; however, other males who had equally extreme cone ratios did not have nucleotide substitutions in the promoter regions. The finding that two males with unusual cone ratios had associated promoter mutations is consistent with previous evidence that promoter/LCR interactions are a factor in determining cone ratio. However, the large range of cone ratio variation that cannot be explained by promoter mutations suggests that the current model in which the identity of an L versus M cone is determined by the permanent formation of an LCR/promoter complex is incomplete. The experiments presented here are a positive step in ultimately understanding the mechanism. From the observations here, we propose that a complete model will have additional genetic components that are involved in determining whether an L or M pigment is expressed in a cone. The variability in cone ratio provides an avenue for identifying these elements by screening for polymorphisms that are associated with the ratio differences. The results here indicate that important genetic regulatory elements that are involved in determining L:M cone ratio are located outside of the region screened in this study. Efforts are underway to screen further upstream of the L and M genes, as well as within the first intron of the L and M genes for genetic polymorphisms that correlate to variation in cone ratio.

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