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L-cone pigment genes expressed in normal colour vision

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Abstract

To directly test the hypothesis that only two pigment genes are expressed from the X-chromosome array, we examined expressed M and L pigment gene sequences from >100 male eye donors. In this sample, there were eight men who expressed high levels of more than one L pigment gene in addition to M pigment genes. The fact that these eyes expressed both L and M pigment genes at significant levels suggests they were from men with normal colour vision. We reject the hypothesis that only two pigment genes from one X-chromosome array can be expressed. © 1998 Elsevier Science Ltd. All rights reserved.

Keywords: Colour vision; Pigment genes; Pigment gene expression; Cone pigment

1. Introduction

The X-chromosome visual pigment gene arrays underlying normal colour vision can be complex, containing multiple genes encoding long-wavelength sensitive (L) pigments as well as multiple genes encoding middle-wavelength (M) pigments [1–4]. The presence of extra genes raises the question of which genes from the array are expressed. The evidence is clear that, in some cases, not all of the genes in an array are expressed [5]. From examples in which expression of specific genes was not detected, it has been hypothesized that significant expression is limited to two pigment genes from each array. The idea that only two genes in the array are expressed has also been forwarded to explain why some deuteranomalous men have, but do not express, M pigment genes.

Efforts to understand the relationship between the genes and colour vision have resulted in disagreement about how many cone pigment genes are expressed in an eye, the levels of expression of different genes within an individual and the extent of variation in pigment gene expression in the population. The present experiments were designed to directly test the hypothesis that an inherent feature of the human X-chromosome pigment gene array is that only two genes are expressed at levels which are significant for vision. We used direct, fluorescent sequencing of photopigment cDNA to screen >100 male eye donors. We examined the majority of the coding sequence, thereby maximizing the probability of detecting variant genes expressed at significant levels.

2. Methods

2.1. Donor tissue and nucleic acid isolation

Human eyes and blood samples were obtained through the Wisconsin Lions Eye Bank. Eyes were enucleated and refrigerated within 5 h of death. Within 24 h of death the retinas were removed and a trephine was used to take a 6 mm punch of retina centered on the fovea. Nucleic acids were isolated from the tissue and genomic DNA was isolated from blood as described previously [6,7].

2.2. L and M pigment cDNA analysis

Retinal RNA was reverse transcribed and the resulting cDNA was used in the polymerase chain reaction (PCR) to selectively amplify exons 2–5 of L or M pigment cDNAs. Hotstart PCR was done using Ampli-

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wax gems and the XL PCR kit (Perkin–Elmer) in reaction volumes of 100 μl containing 1.4 mM magnesium acetate and 30 pmol of each primer. A primer to sequences at the 5′ end of exon 2 (5′CCTTCGAAGGCCCGAATTA) was paired with either an L specific primer (5′GCAGTACGCAAAGATCATCACC) or an M specific primer (5′AAGCAGAATGCCAGGACCATC). The L and M specific primers corresponded to sequences in exon 5 and their selectivity was described previously [4]. The thermal cycling parameters were: 1 cycle at 94°C for 5 min; followed by 35 cycles of 94°C for 1 min, 59°C for 45 s and 72°C for 1 min. The cDNA amplification product spans from exon 2 through exon 5. The cDNA was differentiated from the genomic DNA by the size difference (0.72 kb versus 5.8 kb). For cDNA sequencing, selectively amplified L and M pigment cDNAs were used in hotstart PCR with AmpliTaq Gold (Perkin–Elmer). Primers were the same as those described above except they were tagged with the M13-21mer (M13F) or M13 reverse (M13R) primer sequence. The thermal cycling parameters were: 1 cycle at 95°C for 9 min; followed by 8 cycles of 94°C for 1 min, 59°C for 45 s and 72°C for 1 min; followed by 35 cycles of 94°C for 1 min and 68°C for 45 s. Each PCR product was directly sequenced in both directions with fluorescently labeled M13F or M13R primers in cycle sequencing using AmpliTaq FS (Perkin–Elmer) and an ABI 310 genetic analyzer. To confirm the direct sequencing results in individuals who expressed multiple Ls, PCR products were cloned using the TA-cloning kit (Invitrogen) as described by the manufacturer's instructions. Individual clones were sequenced as described above.

Competitive, quantitative PCR and *Rsa*I restriction digestion analysis was done to estimate the relative ratio of M/L pigment cDNA in each retina. Both primers had a 5′ fluorescein tag; the forward primer sequence was 5′Fl-CCGAGCGGTGGCAAAGCAG and the downstream primer was 5′Fl-TGGCAGCAGCAAAGCATGC. The forward primer corresponds to the 3′-end of exon 4 and 5′ end of exon 5 (spanning intron 4) and the reverse primer corresponds to the 3′ end of exon 5. Amplification conditions were: 1 cycle at 94°C for 5 min; followed by 30 cycles of 94°C for 45 s, 57°C for 45 s and 72°C for 45 s. The amplified cDNA was digested with *Rsa*I, the fragments were separated by gel electrophoresis, visualized with a FluorImager and quantitated. One strand of each heteroduplex is from L pigment cDNA and the other is from M pigment cDNA. Thus, half the signal in the heteroduplexes (H/2) are added to the signal in the uncut band (U) and the other half are added to the sum of the signal in the two cut bands (C). The final quotient: $(U + H/2)/(C + H/2)$ yields an estimate of the ratio of M to L pigment cDNA. This method relies on the heteroduplexes being uncut. If there was a slow rate of cleavage of the heteroduplexes the duration of digestion could affect the estimated cDNA proportions. To insure this was not a problem, samples were digested for durations of 1–3 h. There was no significant change in estimated proportion with digestion time.

Competitive, quantitative PCR and *Fok*I restriction digestion analysis was done to estimate the relative ratios of different L pigment cDNAs in individual retinas. In exon 3, a C/A nucleotide polymorphism in codon 153 produces a restriction site polymorphism for enzyme *Fok*I. Selectively amplified L or M pigment cDNA was used in hotstart PCR with AmpliTaq Gold to amplify exon 3. The forward primer sequence was 5′-GGATCACAGGTCTCTGGTCTCTGG and the reverse primer sequence was 5′-CTGCTCCAACCAAAGATGG. The thermal cycling parameters were: 1 cycle at 95°C for 9 min; followed by 35 cycles of 94°C for 1 min and 68°C for 45 s. The amplified cDNA was digested with *Fok*I and the fragments were separated by gel electrophoresis. Gels were fixed, stained with ethidium bromide [8] and DNA was visualized with the

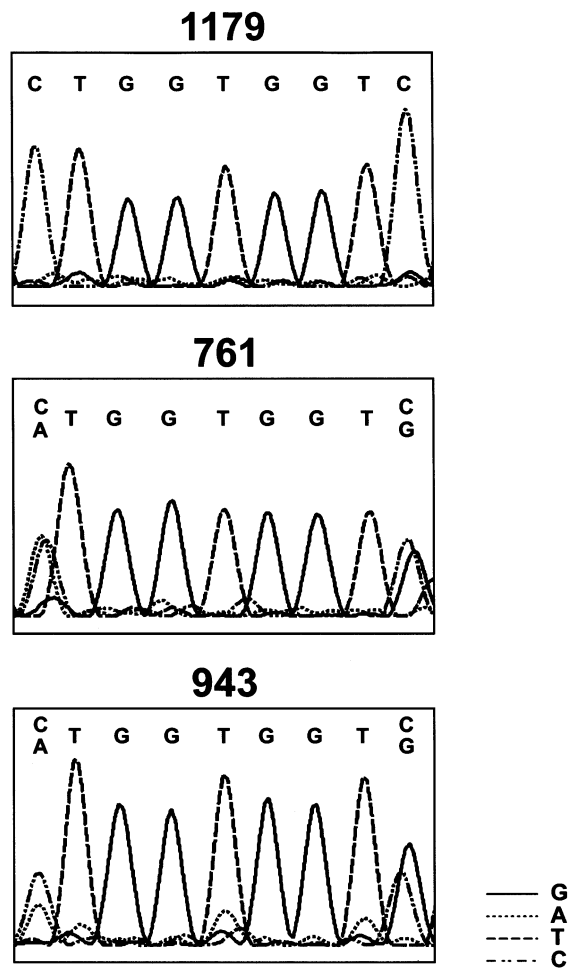

Fig. 1. Sequence electropherograms showing expression of multiple L genes in individual retinas. The nucleotide(s) indicated by the peak(s) at each position is indicated.

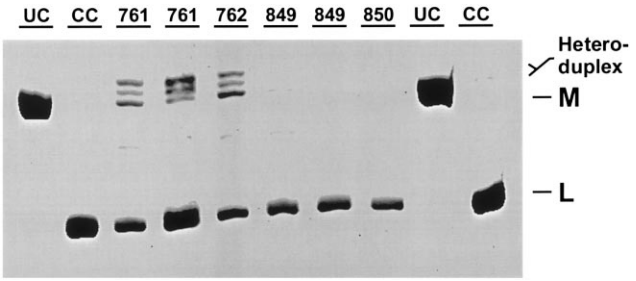

Fig. 2. Fluorimage of competitive, quantitative PCR and *Rsa*I restriction digestion to estimate the relative ratio of M/L pigment cDNA from each retina. Heteroduplexes form when M and L gene fragments are both present. The bands corresponding to the heteroduplexes, M and L gene fragments are indicated. Lanes CC and UC: pigment gene standards with and without the *Rsa*I site, respectively. Lanes 761, 761 and 762 are from left to right, the initial nucleic acid sample, the backup sample and the fellow eye sample, respectively, from donor 761. Lanes 849, 849 and 850: same as for the previous three lanes except nucleic acid is from donor 849, a presumptive deutan, colour blind donor for whom M pigment cDNA could not be detected.

FluorImager. A standard curve for quantitation with this assay was generated using predetermined mixtures of pigment gene standards with and without the *Fok*I site.

2.3. Genomic L and M gene analysis

We estimated the number and ratio of M and L pigment genes in the X-chromosome gene array as described previously [3]. Long distance PCR was done to amplify the first gene in the X-chromosome array [9]. The long-distance PCR product was used as template to amplify exons 2, 3, 4 and 5 for direct, dye-primer sequencing. Primers and conditions were those described by Neitz et al. [4], except that the primers were tagged with the M13 forward or reverse primer sequences for dye-primer sequencing.

All genomic L pigment genes were selectively amplified using the same primers used to selectively amplify L pigment cDNA, but the thermal cycling parameters were: 1 cycle at 94°C for 5 min; followed by 37 cycles of 94°C for 30 s, 59°C for 1 min and 72°C for 5 min and 30 s. The PCR product was gel purified and used to amplify exons 2, 3 and 4 for direct, dye-primer sequencing as described above for the first gene in the array.

2.4. Replicates and contamination precautions

For each donor, retinas were dissected in a building separate from the one housing the molecular biology laboratories. Nucleic acid isolation was done in a room physically distant from the one in which amplified or cloned DNA is handled. All equipment and bench-tops used in isolating DNA and in analyzing amplified DNA were treated with 10% bleach for 30 min after each use, thus rendering contaminating DNA unamplifiable [10].

For each donor, the sample from one retina was split in half. One half was aliquoted and used in initial experiments, the other half was stored at −80°C as a backup. Retinal tissue from the fellow eye was stored at −80°C and nucleic acid was isolated from it at a later time. To monitor for reproducibility and contamination, experiments were done using three independent retinal nucleic acid samples—the initial aliquoted one, the backup and the fellow eye.

3. Results

3.1. Screen for colour normal donors who express more than one L pigment gene

The nucleotide sequences of specifically amplified L and M pigment cDNAs from retinas of 150 male donors were determined. M pigment cDNA was not detected in retinal samples for eight of the 150 donors (5.3%), suggesting that those donors had color vision defects, either deuteranomaly or deuteranopia. This is consistent with the frequency of deutan defects in Caucasian populations, which is about 6% (5% deuteranomaly, 1% deuteranopia). No L pigment cDNA was detected in four of 150 retinas, suggesting that 2.7% of our donors suffered protan defects, consistent with the frequency of protan defects in Caucasian populations. Both M and L cDNA were detected in the remaining 138 retinas. Because these men expressed both L and M pigment genes at significant levels, they were presumed to have had normal color vision.

We obtained clear sequence from exons 2–4 from 100 of the 138 presumptive colour normal retinas. Of these, there was evidence of expression of multiple L pigment genes in eight retinas. By sequencing known ratios of two genes, we experimentally determined that the minor species must be robustly expressed, representing more than 20% of the total cDNA in the mixture being sequenced, to be detected reliably in the electropherogram. The second L cDNA in each of the eight retinas was readily detectable in the electropherograms from sequencing specifically amplified L pigment cDNAs from individual retinas. M pigment cDNAs were also subject to sequence analysis and confirmed the presence of M pigment gene expression in all eight donors. We selected two (donors 761 and 943) of the eight donors who robustly expressed multiple L pigment genes for a detailed analysis of the relationship between expression and the structure of the gene array.

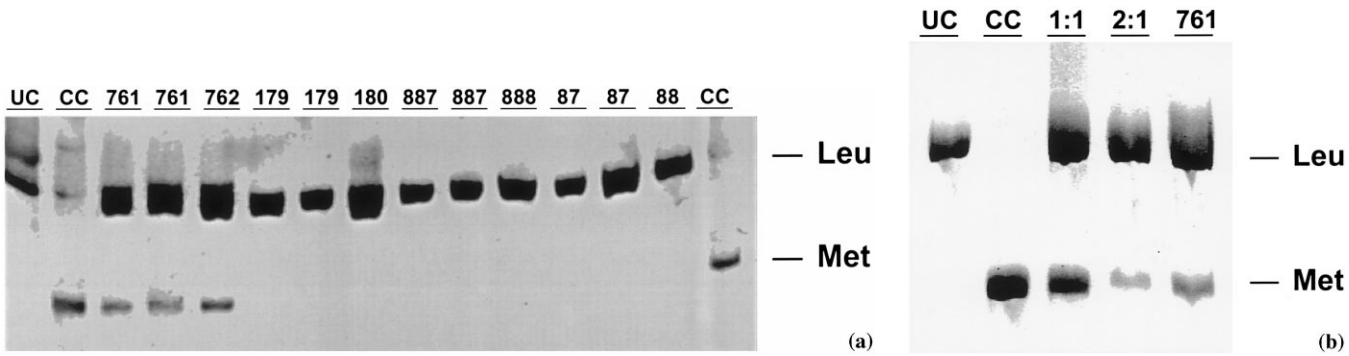

Fig. 3. Fluorimage of competitive, quantitative PCR and *Fok*I restriction digestion to estimate the relative ratios of different L pigment cDNAs in individual retinas. (a) When codon 153 encodes leucine (Leu), there is no *Fok*I site, when methionine (Met) is encoded, there is a *Fok*I site. Lanes CC and UC: pigment gene standards with and without the *Fok*I site respectively. Lanes 761, 761, 762 are from left to right, the initial nucleic acid sample, backup sample and fellow eye from donor 761. This donor clearly expresses two species of L cDNA. The band labeled Leu contains homoduplexes for the L cDNA lacking the *Fok*I site and heteroduplexes between the two different L cDNAs. Lanes, 179, 179, 180; lanes 887, 887, 888; and lanes 87, 87 and 88: are from left to right the initial nucleic acid sample, the backup and the fellow eye samples for donors 179, 887 and 87, respectively. All three had a single L cDNA sequence. (b) Example of results used to estimate the ratio of two L pigment cDNAs. Lane UC and CC: same as in (a). Lanes 1:1 and 2:1 mixtures of pigment gene standards in the indicated ratios used to calibrate the quantitation. Lane 761: example of quantitative assay on nucleic acid from donor 761.

3.2. Detailed analysis of expression in two men

Fig. 1 shows a portion of the sequence electropherograms for donors 761 and 943. A third donor, 1179, for whom a single L pigment cDNA sequence was observed, is shown for comparison. Two peaks at one nucleotide position indicate the presence of more than one L pigment cDNA. The relative peak heights provide a rough estimate of the relative amounts of the different L pigment cDNAs. For donor 761, peak heights were nearly equal at the dimorphic positions, suggesting nearly equal expression of two different L genes. In contrast, for donor 943 one nucleotide peak was higher than the other at the dimorphic positions, suggesting unequal expression of two L genes. This was the more typical result among men who expressed multiple L genes. Cloning and sequencing of cDNA amplified fragments confirmed the presence of more than one L pigment cDNA in both donors 761 and 943.

To estimate the relative ratio of L/M cDNA and of different L pigment cDNAs from donors 761 and 943, we used competitive quantitative PCR and restriction digestion analysis. This was done in triplicate on the initial nucleic acid aliquot for each donor and it was repeated with the backup and fellow eye nucleic acid samples. Results from a minimum of four of the samples were averaged to yield an estimate of the proportions of the two Ls and of the M expression.

Fig. 2 shows a FluorImage of the analysis of the L/M cDNA ratio for donor 761. For each donor, similar results were obtained for the nucleic acid from the initial sample, the backup and the fellow eye. Of the total L and M cDNA, $82 \pm 3\%$ (mean $\pm$ S.E.M.; $n = 4$ independent samples) was L pigment cDNA for donor 761 and $63 \pm 6\%$ (mean $\pm$ S.E.M.; $n = 4$ independent samples) was L pigment cDNA for donor 943. The proportions of L pigment cDNA for these two donors fell well within the range observed for the other 138 donors who were presumed to have had normal colour vision. A band corresponding to the M fragment and the presence of heteroduplexes confirmed the presence of M cDNA.

This analysis was also done with nucleic acid from eight suspected deuteranomalous and deuteranopic donors. As for the one whose results are shown in Fig. 2, no M pigment cDNA was detected for any of the eight deutans even though some of them had M pigment genes. This provides a control to monitor for cross contamination of samples; no contamination was observed.

We estimated the relative ratios of different L pigment cDNAs from the retinas of donors 761 and 943 (Fig. 3). As a control, this measurement was done for three additional donors who, in previous experiments, had a single L pigment cDNA sequence. The analysis was done in triplicate on the initial aliquot of nucleic acid and was repeated later with the backup and fellow eye samples. Two different L pigment cDNA species were observed in all replicates for donors 761 and 943, while a single L cDNA species was detected in all replicates from the three control retinas (Fig. 3a).

For donor 761 the two L pigment cDNA species were estimated to be present in a ratio of 60%:40% and for donor 943 the ratio of two L cDNA species were estimated to be (Fig. 3b). These results were consistent with the peak heights seen in the sequencing electropherograms (Fig. 1).

Table 1
Deduced amino acids encoded by the indicated codons for L genes in genomic DNA and expressed in the retinas (cDNA) from two donors

	Exon 2			Exon 3					Exon 4		
Codon no.	65	111	116	153	171	174	178	180	230	233	236
Donor 761											
(A) L pigment cDNA	T/I	I/V	S/Y	L/M	I/V	A/V	I/V	S/A	I/T	A/S	M/V
(B) 1st gene	T	I	S	L	I	A	I	S	I	A	M
(C) all genomic L genes	T/I	I/V	S/Y	L/M	I/V	A/V	I/V	S/A	I/T	A/S	M/V
(D) second L derived from rows B and C	I	V	Y	M	V	V	V	A	T	S	V
Donor 943											
(A) L pigment cDNA	T/I	I/V	S/Y	L/M	V	A/V	I/V	S/A	I	A	M
(B) 1st gene	T	I	S	L	V	A	I	S	I	A	M
(C) all genomic L genes	T/I	I/V	S/Y	L/M	V	A/V	I/V	S/A	I	A	M
(D) second L derived from rows B and C	I	V	Y	M	V	V	V	A	I	A	M

Locations with two amino acid codes are sites where sequences corresponding to both codons were seen in the sequencing electropherogram. A, alanine; I, isoleucine; L, leucine; M, methionine; S, serine; T, threonine; V, valine; Y, tyrosine.

3.3. Structure of the X-chromosome array

Number and ratio X-chromosome pigment genes. The number and ratio of X-linked pigment genes was estimated for donors 761 and 943 using competitive quantitative PCR and *Rsa*I restriction digestion analysis. Estimates were determined as the average of triplicate experiments. For donor 761 the number of X-linked pigment genes was 3.6 ± 0.54 (mean $\pm$ S.E.M.) and the ratio of M/L genes was 0.99 ± 0.01 (mean $\pm$ S.E.M.). From these values, we estimate that donor 761 had an array with 2 L and 2 M pigment genes. For donor 943 the number of X-linked pigment genes was 6.4 ± 0.07 (mean $\pm$ S.E.M.) and the ratio of M/L genes was 2.87 ± 0.21 (mean $\pm$ S.E.M.). Thus, donor 943 had an array with an estimated 2 L and 5 M pigment genes.

3.4. Relationship between the structure of the array and expression

Expressed L pigment sequences. A total of 11 dimorphic amino acid positions are encoded by exons 2–4 of the pigment genes. In the sequencing electropherograms for the L pigment cDNAs from donor 761, there were two peaks of nearly equal height at nucleotide positions within codons specifying all 11 dimorphic amino acid positions. This is indicated in Table 1 (donor 761 row (A)). The amino acids at the 11 dimorphic positions specified by L pigment cDNAs from donor 943 are also shown in Table 1. Recall that the nucleotide peak heights (Fig. 1) indicated unequal expression of the different L pigment cDNAs for donor 943. From the relative peak heights we deduced the amino acids specified by the more versus less abundant L cDNA. The smaller font letters in Table 1 indicate the amino acid species that had relatively lower abundance, row (A) for donor 943.

Genomic sequences. The unique sequences upstream of the first gene in the array make it possible to amplify and sequence the first gene separately from the other genes in the array [9]. The sequence of exon 5 indicates whether the gene encodes an M pigment or an L pigment [11–13]. In L pigment genes, codons 277 and 285 specify tyrosine and threonine, respectively. In both donors, an L pigment gene was first in the array. The amino acids specified at each of the 11 dimorphic amino acid positions encoded by the first gene in the array for each donor are shown in Table 1, rows (B).

For each donor, we also determined the nucleotide sequence of exons 2–4 of both genomic L pigment genes together in a mixture. Recall that both donors were estimated to have 2 genomic L genes. The amino acids encoded at the 11 dimorphic positions deduced from the DNA sequence of a mixture of both L genes for each donor are shown Table 1, rows (C). We deduced the sequence of the second L gene by comparing the sequence from the first gene to the sequence from both L genes in each array. The deduced amino acids specified at each of the dimorphic positions for the second L gene in each array are shown in Table 1, rows (D).

3.5. Summary

Analysis of cDNA and genomic DNA revealed: (1) each donor had 2 L pigment genes, this was independently confirmed by three methods; (2) for both donors, an L gene was first in the array; (3) the deduced sequences of the genomic L pigment genes corresponded in sequence to the L pigment cDNAs from the retinas of each donor; (4) the first gene in each array was the one that was expressed the most.

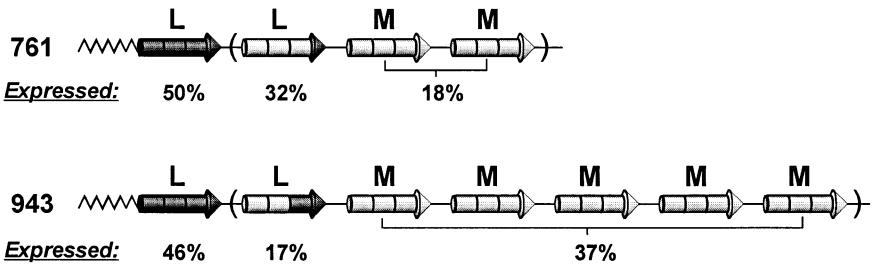

Fig. 4. Relationship between gene array and gene expression for two donors. The identity of the first gene in each array is known. The order of the downstream L and M genes is not known and is drawn arbitrarily indicated by parenthesis. Of the total L and M pigment cDNA from each donor retina, the percentage contributed by each L gene in the array is indicated. We do not know which M genes are or are not expressed. Therefore, the percentage of total X-linked photopigment cDNA contributed from M genes is indicated with a bracket encompassing all M genes.

4. Discussion

Direct sequencing of L pigment cDNA from the retinas of 100 male eye donors who expressed both L and M pigment genes revealed that eight men robustly expressed 2 L genes. Experimental data from the detailed analysis of the X-linked pigment gene arrays and the expressed L pigment genes for two such donors is the basis for a model of the relationship between expression and the structure of the X-chromosome visual pigment gene array shown in Fig. 4. The first gene in each array encoded an L pigment and it was the gene that was expressed the most. Each array had a second L gene, which was also expressed robustly. In addition, both donors had robust expression of M genes.

The second 'L' pigment gene in each of these subjects had a structure (Fig. 4) that would classify it as a 'fusion' or 'hybrid' gene according to Nathans et al. [14]. Such genes are now understood to be associated with normal color vision as well as color vision defects [15,1,3]. Evidence presented here indicates that 'hybrid' genes are not only present but they are expressed in normal color vision. It is also clear from earlier studies that in exons 2, 3 and 4 of the pigment genes, there are frequent polymorphisms that produce spectral subtypes both in normal and color defective vision [4,16]. All these factors blur the distinction between normal and hybrid genes. One approach that avoids these problems is to simply classify X-encoded pigments into two main classes, M and L, according to their spectral peak. The expressed 'M/L hybrid' genes from these two, presumably color-normal eye donors have predicted peaks of about 551 and 555 nm [11] placing them in the L class.

The data refute the hypothesis that only two genes from the X-chromosome pigment gene array are expressed. The related hypotheses that only one type of M and one type of L pigment gene are expressed, or that only two genes are expressed at significant levels can be similarly laid to rest. If the relative levels of expression determine the relative amounts of functional pigments, then all of the robustly expressed pigments would be expected to significantly contribute to colour vision.

It has been suggested that if only the first two genes from each array are expressed this would explain the presence of M genes that do not produce functional M pigment in arrays of some deuteranomalous men. The addition of another L gene in the second position in the array was proposed to displace the M pigment genes to a non-expressed position. The loss of M pigment function is the defect in deuteranomaly. Clearly more than two genes in an array can be expressed, leaving the genetic defect responsible for most cases of deuteranomaly an open question. One possibility is that deuteranomals have M pigment genes with specific mutations that interrupt expression, or function of the M pigment, or the viability of the M cones.

So 8% of the presumptive colour normal donors studied here robustly expressed multiple L pigment genes. The present experiments were not designed to detect L genes expressed at less than 20% of the total L gene expression and they cannot detect cases where two identical L genes are expressed. Thus, very likely more than 8% of the color normal male population express more than one L gene at a level that could significantly contribute to colour vision.

Acknowledgements

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