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Expression of L cone pigment gene subtypes in females

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Abstract

Spectral subtypes of L pigment are produced by a serine/alanine dimorphism at amino acid position 180. X-chromosomes that carry genes for the different subtypes occur with about equal frequency in normal men. Females have two X-chromosomes; thus, about 50% of women will inherit genes for both L pigment subtypes, although on different X-chromosomes. In these women, X-inactivation is expected to produce about equal numbers of L_{S180} and L_{A180} cones in addition to middle (M) and short (S) wavelength-sensitive cones to total four spectrally distinct cone types. Consistent with this expectation we found nearly equal expression of genes for two spectrally distinct subtypes of L pigment in five of nine female retinas examined.

Keywords: Cone pigment gene expression; Gender differences

1. Introduction

There are spectral subtypes of long-wavelength sensitive (L) cone pigments in the colour normal male population. The most commonly observed, spectrally active amino acid dimorphism is the one at amino acid position 180 [1–3]. The difference of serine (L_{S180}) versus alanine (L_{A180}) makes a 4–7 nm difference in the spectral peak of the pigment [4–8].

The frequency of colour normal men who predominantly express a gene for L_{S180} versus L_{A180} was estimated from a study of X-linked pigment cDNAs from the retinas of 100 male eye donors [9]. The predominantly expressed L pigment gene encoded serine180 in 51.5% of the men and it encoded alanine180 in 48.5% of the men (95% confidence limits $\pm 8.5\%$). These values are consistent with earlier estimates of the frequencies of X-chromosomes with these genes [2,10,11].

In females, early in embryonic development, each cell inactivates either the maternal or paternal X-chromosome [12]. As the cells continue to divide after X-inactivation has occurred, each daughter cell maintains the same inactivated X as the mother cell from which it derived. Which X-chromosome is inactivated in any given cell is thought to be randomly determined, and thus about half of the cells in the embryo inactivate the maternal X and the other half inactivate the paternal X-chromosome. These observations lead to the prediction that the structure of the photoreceptor mosaic in many females will be quite different than it is in men. The M/L cone photoreceptors in the female retina are expected to form a mosaic in which about half of the cones express visual pigment genes from the maternal X-chromosome array, and the other half express genes from the paternal array. The frequency of women who are expected to have nearly equal populations of L_{A180} and L_{S180} cones is near 50% ($2 \times 0.515 \times 0.485$). Thus, about half of all women are expected to have at least four spectrally distinct cone types, including nearly equal numbers of L_{S180} and L_{A180} cones plus M and S cones.

This expectation follows directly from well-established facts of normal polymorphism in the cone pigments and of X-inactivation. Yet, it represents a picture of the cone mosaics of normal women that differs sharply from the traditional idea that, with the exception of heterozygous carriers of colour anomaly, all women have three cone types. It, thus, seems worthwhile to seek direct experimental evidence in support of

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Table 1
PCR primers

Primer	Location	Specificity	Sequence[a]
1	Exon 2, 5′ end	L & M	5′CCTTCGAAGGCCCGAATTA
2	Exon 5, middle	L only	5′GCAGTACGCAAAGATCATCACC
3	Exon 2, 3′ end	L & M	5′tgtaaaacgacggccagtCCTTCGAAGGCCCGAATTA
4	Exon 3, 5′end	L & M	5′caggaaacagctatgaccCTGCTCCAACCAAAGATGG
5	Exon 4, 5′end	L & M	5′tgtaaaacgacggccagtTACTGGCCCCACGGCCTGAA
6	Exon 4, 3′ end	L & M	5′caggaaacagctatgaccTCGGATGGCCAGCCACACTT
7	Exon 3, 5′end	L & M	5′FLGGATCACAGGTCTCTGGTCTCTGG
8	Exon 3, 3′end	L & M	5′FLCTGCTCCAACCAAAGATGG
9	Exon 4, middle	L only	5′CATGATGATAGCGAGTGGGATG
10	Exon 4, middle	M only	5′CACGATGATGCTGAGTGGGGT

[a] Lower case letters indicate M13F and M13R sequence tags. FL indicates a 5′ fluorescein tag.

the theory. It is possible to gain information about cone pigment gene expression by examining cone pigment messenger RNA (mRNA) from the retinas of female eye donors. The theory predicts a high frequency of female eye donors will show evidence of nearly equal expression of two subtypes of L pigment genes in addition to M and S.

2. Methods

2.1. Donors, tissue and nucleic acid isolation

Human retinas were obtained from nine female eye donors. Retinal tissue samples were isolated and frozen within 5 h of death. For four donors (F22, F25, F34 and F36), a 4×20 mm strip of far peripheral retina, near the ora serrata was obtained. Samples from these same four donors were used in earlier experiments which correlated cone pigment amino acid differences with the spectra of individual cones recorded electrophysiologically [8]. From the other five donors (F1237, F1585, F337, F419, and F445), a trephine was used to take a 6 mm punch of retina centered on the fovea. Retinal tissue was frozen, and stored at -70°C. Retinal nucleic acid was isolated as described previously [13].

2.2. Reverse transcription, polymerase chain reaction, DNA sequencing, and restriction digestion analysis

Retinal RNA was reverse transcribed and the resulting cDNA was used in the polymerase chain reaction (PCR) to selectively amplify L pigment cDNA. Hotstart PCR was done using Ampliwax gems and the XL PCR kit (Perkin-Elmer) in a reaction volume of 100 μl containing 1.4 mM magnesium acetate and 30 pmol of each primer. Primer sequences are given in Table 1. The thermal cycling parameters were 94°C for 5 min for 1 cycle; 94°C for 1 min, 59°C for 45 s, 72°C for 1 min for 30 cycles; 72°C for 10 min for 1 cycle. Primers 1 and 2 were paired to selectively amplify L pigment cDNAs. The selectivity of primer 2 was described previously [10].

For cDNA sequencing, selectively amplified L pigment cDNAs were used in hotstart PCR with AmpliTaq Gold (Perkin-Elmer) and visual pigment cDNA specific primers tagged with the M13-21mer (M13F) or M13 reverse (M13R) primer sequence. Primers 3 and 4 were paired to amplify exons 2 and 3, and primers 5 and 6 were paired to amplify exon 4. The thermal cycling parameters for primers 3 and 4 were 94°C for 9 min for 1 cycle; 94°C for 1 min, 59°C for 45 s, 72°C for 45 s for 30 cycles; 72°C for 10 min for 1 cycle. These same conditions were used for primers 5 and 6 except that instead of 59°C for 45 s, the temperature was 61°C. Each PCR product was directly sequenced in both directions with M13F or M13R primers in dye-primer cycle sequencing using AmpliTaq FS (Perkin-Elmer) and an ABI 310 genetic analyzer.

The relative ratio of cDNA for L_{S180} versus L_{A180} in each donor retina was estimated using competitive quantitative PCR and restriction digestion analysis. Selectively amplified L pigment cDNA was used in PCR to amplify exon 3 with primers 7 and 8, and exon 3 was digested with restriction enzyme Fnu4H I. The thermal cycling parameters were 94°C for 5 min for 1 cycle; 94°C for 1 min, 68°C for 45 s for 30 cycles; 72°C for 10 min for 1 cycle. When alanine180 is specified, there is a Fnu4HI restriction enzyme cleavage site that is absent when serine180 is specified. The restriction digestion fragments were electrophoretically separated, visualized with a FluorImager, and the amount of DNA in each band was measured using ImageQuant software (Molecular Dynamics). The relative ratio of the L pigment cDNAs differing with regard to the presence or absence of the Fnu4H I site was estimated by comparison with results from a standard curve constructed using known ratios of L pigment cDNA.

Selective amplification of subtypes of L pigment cDNA was done using retinal samples for two subjects, F22 and F337. L pigment cDNA obtained with primers 1 and 2 was used in PCR with primers 1 and 9, or primers 1 and 10 to selectively amplify the subset of L pigment cDNAs that contained a typical L pigment exon 4 sequence, or a typical M pigment exon 4 sequence, respectively. The specificity of primers 9 and 10 have been described previously [7,10]. The PCR products were sequenced directly, as described above, to obtain the DNA sequence of the typical L and M/L hybrid cDNAs, separately. For donor 337, we amplified the first gene in each X-chromosome array using a primer selective for the first gene. This primer was paired either with a primer selective for L gene exon 4 or M gene exon 4. The primers and the PCR conditions used have been described elsewhere [14].

3. Results

The nucleotide sequences of exons 2, 3 and 4 of selectively amplified L pigment cDNAs from the retinas of nine females were determined. Examples of the sequencing data are shown in Fig. 1, which shows portions of the electropherograms for exon 3 of the L pigment cDNAs from the retinas of female donors F34 (top panel, Fig. 1) and F445 (bottom panel, Fig. 1). Two peaks in one location in the electropherogram indicate two different nucleotides were present at one position and that there were at least two different cDNA species present. In the top panel in Fig. 1, there are two peaks corresponding to the first nucleotide position in codon 180. Consequently, the encoded L pigments differ in whether there is serine or alanine at amino acid position 180, and thus they differ by 4–7 nm in λ_{max}. Since dye-primer sequencing chemistry was used, the relative peak heights of the two nucleotides at one position provided a rough indication of the relative amounts of the cDNAs differing at that site that were present in the sequencing reactions [15]. We have confirmed that this sequencing method yields a qualitative estimate of the relative amounts of mRNA by testing known ratios. Nearly equal peak heights for the two nucleotides in the first position of codon 180 suggested that genes encoding L_{S180} and L_{A180} were expressed in similar amounts. This was observed for five (F22, F34, F337, F419 and F445) of the nine female donors. For some donors, peaks of equal height were observed at other known polymorphic sites within the L pigment cDNAs, and this provided additional evidence that roughly equal amounts of two different sequence variants of L pigment were expressed in the individual female retinas. An example is shown in the lower panel of Fig. 1, in which two peaks were observed at nucleotide positions within codons 151 and 153 in exon 3 of the L pigment cDNAs from donor F445.

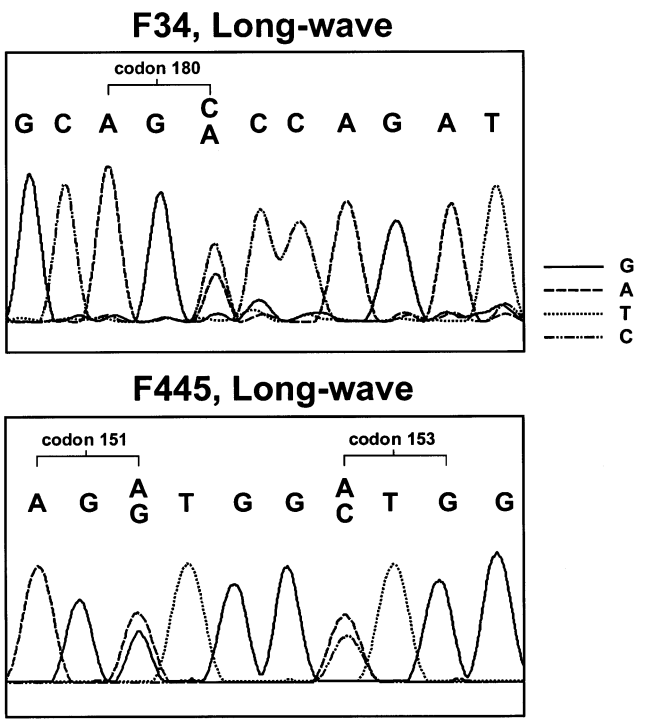

Fig. 1. Sequence electropherogram for exon 3 of L pigment cDNAs from donor F34 (top panel) and donor F445 (bottom panel). The nucleotide peak(s) detected at each position in the sequence are indicated at the top. Codons in which one nucleotide position contained two peaks are indicated. The top panel shows the negative strand sequence. The coding sequence is the reverse complement of that shown. The first nucleotide of codon 180 is, thus, in the right most position. The bottom panel shows the positive strand.

Partial amino acid sequences of the L pigments expressed in individual retinas were deduced from the sequences of exons 2, 3 and 4 of the cDNAs. Exons 2–4 encode 11 amino acid positions that are dimorphic among and between the L and M photopigments. Table 2 summarizes the amino acids specified at these 11 positions by the L pigment cDNAs from the donor retinas.

Five of nine women examined expressed genes for at least two spectrally distinct L pigments, L_{S180} and L_{A180} (Table 2). We used competitive quantitative PCR and a Fnu4HI restriction digestion assay to estimate the relative proportion of cDNAs encoding L_{S180}/L_{A180} for these donors. As an illustration, results for five donors are shown in Fig. 2. For two donors, F1237 and F1585, only bands corresponding to L_{S180} pigment cDNA were observed, thus confirming the DNA sequencing data (Table 2). FluorImage analysis of the gel revealed that the cDNAs for L_{S180} and L_{A180} were present in about equal amounts in each of the donor retinas, F22, F34, F337, F419, F445. This is consistent with the results observed in the sequencing electropherograms, where, in each case, the nucleotide peaks in the first position of codon 180 were of similar height. Also consistent with

Table 2
Amino acid polymorphisms encoded by L pigment cDNAs

Retinal sample location	Donor no.	Exon 2			Exon 3					Exon 4		
		65	111	116[a]	153	171	174	178	180[a]	230[a]	233[a]	236
Peripheral	F22	T	I/V	S/Y	L	V	A/V	I/V	A/S	T/I	S/A	V/M
Peripheral	F25	T	I	S	L/M	V	A	I	A	I	A	M
Peripheral	F34	T	I	S	L	V	A	I	A/S	I	A	M
Peripheral	F36	T	I	S	L	V	A	I	S	I	A	M
Central	F1237	T	I	S	L	V	A	I	S	I	A	M
Central	F1585	T	I	S	L	V	A	I	S	I	A	M
Central	F337	T	I	S	L	V	A	I	A/S	T/I	S/A	V/M
Central	F419	T	I	S	M	V	A	I	A/S	I	A	M
Central	F445	T	I	S	L/M	V	A	I/V	A/S	I	A	M

I, isoleucine; V, valine; Y, tyrosine; M, methionine; A, alanine; S, serine; L, leucine; T, threonine.
[a] Spectrally active positions [5].

the sequence data, the other four donors showed evidence for a single cDNA species with respect to codon 180.

The DNA sequence data suggested that donors F22 and F337 each express one L pigment gene with an exon 4 sequence that is more typical of M pigment genes. For each of these donors, primers selective for the exon 4 sequence differences were used to separately amplify the different L pigment cDNAs and they were directly sequenced. Donor F22 expressed a gene which could be considered to be a hybrid, containing typical M pigment gene sequences in exons 2, 3 and 4, although L pigment gene sequences in exon 5. Thus, donor F22 may have been heterozygous for a deutan colour vision deficiency. However, hybrid genes, like this one, can also be expressed in men with normal colour vision [9]. For donor F445, one gene contained sequences typical of L pigment genes in exons 2, 3 and 5, but contained typical M pigment gene sequences in exon 4. Genes with this type of structure have been frequently reported for men with normal colour vision [2,10,11].

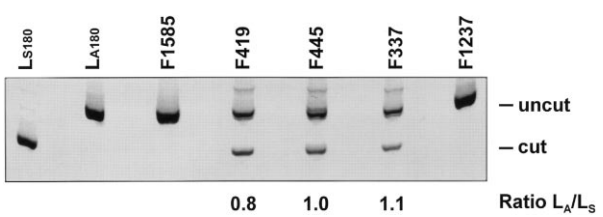

Fig. 2. Fnu4 HI restriction digestion assay to estimate the relative amounts of cDNAs encoding L_{S180} and L_{A180} in individual female retinas. Right most two lanes are controls showing expected bands for L_{A180} and L_{S180}, respectively. Donor numbers are given above each lane. For the donors who showed expression of both L_{A180} and L_{S180} estimated ratios of mRNA expression are given below the lane. The estimates represent the average of two independent determinations but only one is shown in this figure. The estimates were determined by referring to a standard curve. The upper band appears more intense even though the quantitation indicates nearly equal expression (ratios near 1.0). This is, in part, because the smaller cut fragment that runs further down on the gel was cut off in this photo to save space.

For each donor, M pigment cDNAs were also sequenced (data not shown). The M pigment cDNA sequences obtained from donor F36 suggested that she had a hybrid M pigment gene that has been characteristically found in men with protan colour vision deficiency. We used genomic DNA from donor F36 in a recently developed assay designed to identify female carriers of protan colour vision defects [14]. The results indicated that she was a carrier of protan colour vision deficiency.

4. Discussion

Of nine female retinas examined, five expressed genes for at least three spectrally distinct X-encoded cone pigments. For each of the five, we found roughly equal amounts of cDNAs for L_{S180} and for L_{A180} pigment. These results are consistent with the idea that nearly half of women have about equal numbers of L_{S180} and L_{A180} cones as is predicted from the frequency of these subtypes in men.

Men with normal colour vision can have [10] and express [9] genes for more than just two X-encoded cone pigments. However, in the presumed colour-normal male retinas, the expression of one L pigment gene always predominated over both M pigment gene expression and over the expression of any additional L pigment genes. Although several males in our sample of 100 expressed a second L pigment gene at significant levels few approached the nearly equal levels that were estimated for the women examined here.

Polymorphisms of the normal X-encoded pigments in males together with X-inactivation in females predict a major gender difference in the cone populations. In humans, on average, the L cones are estimated to outnumber M cones by a factor of about 2. Thus,

females, who are heterozygous at L pigment position 180 and produce about equal numbers of the two L cone subtypes will have three cone populations of about equal size (L_{S180}, L_{A180} and M) and presumably a fourth smaller population, the S cones. The results here, compared with those of Sjoberg et al. [9] are consistent with there being a major difference between males and females as predicted. Such differences in the retinas of males and females could be the basis for gender differences in colour matching [16,17].

One donor, F36, was determined to have been a heterozygous carrier of a protan colour vision defect and a second, F22, may possibly have carried a deutan colour defect. This is not surprising. The estimated frequency of carriers of colour vision defects is approximately 15% of females; thus, in a sample of nine women one would expect one to two carriers. Of the remaining seven, presuming they all had S cones, four expressed genes for four spectrally different cone pigments. The three pigment hypothesis suggests that humans are normally trichromatic because there are three cone photopigments in the retina [18]. Female carriers of anomalous trichromacy have long been believed to express genes for four different cone pigments. They, thus, have been thought to have unique potential for tetrachromacy. Normal, noncarrier, women have served as controls in experiments to look for tetrachromacy in female carriers, and the normal controls have not been reported to exhibit tetrachromacy [19,20]. This suggests that trichromacy is not imposed by the limitation of the number of spectrally distinct cone pigments to three.

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