Interindividual and topographical variation of L:M cone ratios in monkey retinas

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We analyzed the ratio of L:M cone photopigment mRNA in the retinas of Old World monkeys, using the method of rapid polymerase chain reaction–single-strand conformation polymorphism. The L:M cone pigment mRNA ratio in whole retina ranged from 0.6 to 7.0, with a mean of ~1.6 (standard deviation, ±0.56; $n = 26$). There was no change in this ratio with eccentricity up to 9 mm (~45°), though the ratio was ~30% greater in temporal than in nasal retina. The mRNA ratios are in good agreement with the L:M cone ratio in these same retinas, inferred from electrophysiological recordings of cone signal gain in horizontal cell interneurons. The correlation between mRNA ratios and physiological cone gain ratio supports the conclusion that both measures reflect the relative number of L and M cones. © 2000 Optical Society of America [S0740-3232(00)01403-4]

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1. INTRODUCTION

Trichromatic color vision among primates is based on the presence of three classes of cone photoreceptor: blue [short-wavelength (S)], green [middle-wavelength (M)], and red [long-wavelength (L)] sensitive. The determination of the relative numbers and of the spatial distribution of different cone photoreceptor types in the primate retina is fundamental to our understanding of visual sensitivity and color vision. Selective staining of blue cones has allowed a direct determination of their numbers and distribution.[1–3] While a lack of selective staining has prevented such a direct determination for L and M cones, estimates of their numbers and distribution have been derived by a variety of methods.

In primates a combined L and M cone signal forms the luminance channel in the visual pathway. The psychophysically determined human photopic luminosity function is well modeled as the weighted sum of L and M cone signals.[4] While the mean relative L:M cone contribution to the luminosity function is ~2:1, the ratio varies greatly across individuals. This variability has been observed in a wide number of psychophysical studies.[5–10] Estimates of the L:M cone ratio in human subjects by flicker-photometric electroretinography (ERG) have also shown wide variability in L:M ratio. In one study the L:M cone ratio varied from 0.67 to 9, with a mean of 2,[11] and in another study the ratio ranged from 0.28 to 8.67 with a mean of 4:1.[12] We previously observed that the ratio of L:M mRNA present in human retinas varies widely, ranging from 1 to 10 with a mode of 4.[13] In another study an average L:M pigment mRNA ratio of 1.5 was observed in central human retinal patches, which increased to ~3 for patches at 40° eccentricity.[14] Microspectrophotometric measurements of eyes of individuals with normal color vision revealed an average L:M cone ratio of 1.4 in the foveal region.[15] Psychophysical observations of vernier acuity were used to infer a random arrangement of L and M cones (more L than M) in the human fovea.[16] Finally, the first images of the arrangement of cones in the living human eye revealed an L:M cone ratio of 1.15 in one male subject (selected for a low ratio based on ERG measurements) and an L:M cone ratio of 3.79 in a second, unselected male subject.[17] The distribution of L and M cones in the subject with the 3.79 ratio was not significantly different from random distribution. The L and M cones of the subject with a ratio of 1.15 were, however, significantly more clumped than would be expected from random distribution, though a deviation from random distribution could have resulted from residual optical blur in the subject's retinal images.

In nonhuman primates estimates have generally indicated approximately equal numbers of L and M cones,[18–20] though one exception may be the baboon retina, in which M cones appear to outnumber L cones.[21]

In the macaque monkey, ERG suggests a mean L:M cone ratio of 1:1.[22] Luminance-coding ganglion cells in the macaque, however, have a spectral sensitivity that matches the human photopic luminosity function, which would suggest an L:M cone ratio of 2:1.[23,24]

In this paper we report the L:M pigment mRNA ratios in whole retinas of three species of macaque monkey and one baboon. In several retinas we also examined the relation between the L:M pigment mRNA ratio and retinal topography by analyzing small tissue samples from known retinal locations. Finally, we compared our pigment mRNA ratio measurements with a physiological measure of the functional L:M cone ratio at the level of the horizontal cells, second-order interneurons that receive convergent input from the L and M cone population.[25] The results showed that, for the nonhuman primates sampled, variation in the L:M mRNA ratio was large and was comparable with that observed in the human retina. We also found a nasal–temporal asymmetry, with a reduced L:M ratio in the nasal quadrant. There was a strong correlation between the physiological and the mRNA estimates of the L:M ratio, suggesting that the two data sets are dictated by the relative numbers of L and M cones in the sample.

2. EXPERIMENTAL METHODS

A. Subjects and Retinal Specimens

Monkey retinas were obtained through the tissue distribution program of the Primate Center of the University of Washington, Seattle, Washington. The animals were unselected, did not come from the same breeding colony, and therefore were not closely related. They were free from any obvious ocular pathologies. The entire retina was dissected free of the vitreous and sclera within 1 h of death and was quickly frozen on dry ice. Punches (3 mm in diameter) from various regions of some retinas were quickly frozen on dry ice and were kept at −70 °C until they were used for RNA extraction. In some cases the retinas were kept alive for electrophysiological estimation of L and M cone ratios as described in Ref. 26 before specimens were taken for RNA analysis.

B. Extraction and Analysis of RNA

Total cellular RNA was extracted from monkey retinal tissue by the Trizol method (Gibco–BRL) and was reverse transcribed with a mixture of random hexamer primers as described in Ref. 27. First, strand cDNA was used as a template to competitively amplify, by polymerase chain reaction (PCR), segments (Fig. 1B, 215 base pairs) of the L and M pigment cDNA that encompass the 3′ region of exon 4 and the 5′ region of exon 5. The sequence of primers used in amplification completely matched both L and M pigment mRNA sequences of all primate species examined (Fig. 1B). Three determinations were made on three aliquots from whole retinal or from retinal patch RNA preparations.

The design of the amplification primers was based on previously determined partial coding sequences of exons 4 and 5 of L and M cone pigments of Old World monkeys.[28] We used this information to complete the sequences of the L and M exons 4 and 5 of *Macaca fascicularis* and *M. nemestrina* and designed primers to PCR amplify a segment encompassing the 3′ region of exon 4 and the 5′ region of exon 5, as shown in Fig. 1.

Primer 148 was radiolabeled at its 5′ end with ^{32}P and T4 polynucleotide kinase and was used in the PCR reaction as described in Ref. 13 such that only the sense strand of the amplified products would be detectable by autoradiography after gel electrophoresis. After an initial denaturation step of 2 min at 94 °C, amplification was performed for 25 cycles of denaturation at 94 °C for 15 s,

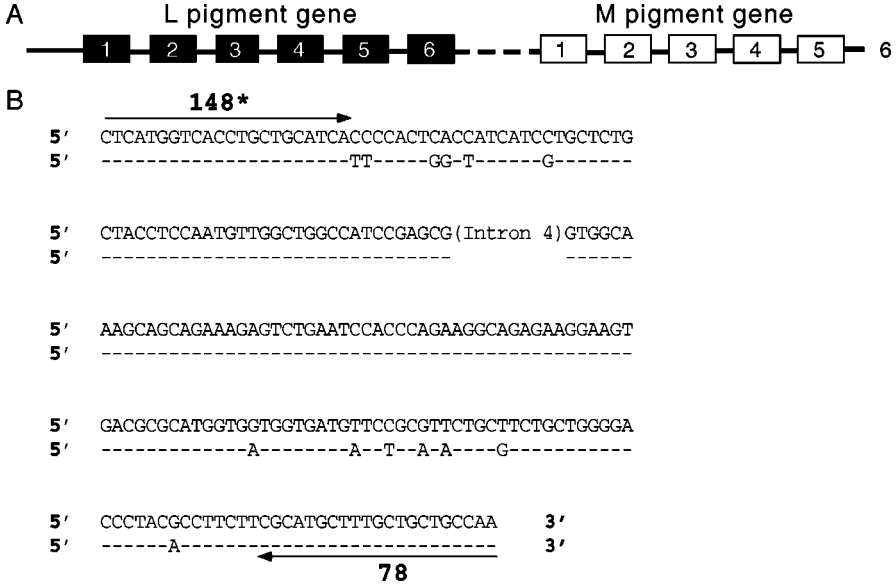

Fig. 1. Strategy for amplification of the L and M exon-4–exon-5 coding region. A, Diagram of the X-chromosome-linked L–M visual pigment gene array. The numbered rectangles represent exons, and the solid lines connecting them represent introns. Diagram is not to scale. B, Sequence of the (top) M and (bottom) L segments that encompass the 3′ end of exon 4 and the 5′ end of exon 5, amplified with primers 148 and 78 (indicated by arrows). The dashes indicate identity in sequence between the L and the M segments. The position of intron 4 is indicated, but, because cDNA was used as the template, only exonic sequences are amplified.

followed by annealing and extension for 30 s at 64 °C. A final extension step was performed at 72 °C for 4 min. The PCR-amplified products were denatured, and the single strands corresponding to the L and M pigments (which differ in nucleotide sequence at a few positions) were resolved by electrophoresis on a nondenaturing polyacrylamide (6%) gel (20 cm × 43 cm) as described in detail elsewhere.[13] Electrophoresis was run at a temperature of 36 °C for ~3 h. Quantification of the bands was performed by phosphorimage analysis. Because they differ in sequence (Fig. 1), the single strands of the L and the M mRNA-derived fragments assume different conformations and differ in electrophoretic mobility.

C. Correlation with Single-Cell Physiology

Horizontal cells were recorded intracellularly in an *in vitro* whole-retina preparation. The weights of L and M cone inputs to each horizontal cell were determined from the contrast gain of responses to sinusoidal flicker in different directions in color space. Detailed methods for cell identification, recording, light stimulation, data acquisition, and analysis of the L and M cone input gain to horizontal cells are given in a related paper[25] in which the main body of physiological results is presented. After the recordings were completed, retinal punches 3 mm in diameter centered on the location of the recorded cells were removed and were placed on dry ice until RNA extraction was started.

3. RESULTS

A. L:M Cone Pigment mRNA Ratios in Whole Retinas

First we constructed a standardization curve that related expected and observed ratios of L:M exon 4–5 sequences. Cloned and purified DNA templates that contained artificial mixtures of known ratios of purified L:M exon 4–5 sequences (labeled Input Ratios in Fig. 2) were used as templates in the PCR amplification reaction. An autoradiograph of the gel that we used to separate L from M segments and to determine their (observed) ratio is known in Fig. 2A. This assay gave a linear response of input and observed values over a wide range (0.25–8) of L:M ratios (Fig. 2B).

Next we determined the L:M mRNA ratio in whole retinas of 26 monkeys: 21 *Macaca nemestrina* (7 males, 14 females), 2 *M. fascicularis* (females), 2 *Papio anubis* (males), and a female *M. mulatta*. An autoradiograph of a single-strand conformation polymorphism gel showing separation of single strands derived from L and M exon 4–5 sequences is shown in Fig. 3A. The L:M mRNA ratios (Fig. 3B) ranged from 0.66 to 7.0, with a mean, ±1 SD of 1.80 ± 0.60 (mean of 1.59 ± 0.56, excluding that of monkey 6, which had a ratio of 7). The frequency distribution of the ratios is given in the inset of Fig. 3B. As was observed in human retinas,[13] the L pigment gene is expressed at higher levels than the M pigment gene in the majority of retinas. There was no statistically significant difference in the L:M mRNA ratio between male (1.93) and female (1.77) monkeys.

B. Topographic Distribution of the L:M Cone Pigment mRNA Ratio

Next we determined the L:M cone pigment mRNA ratio in retinal punches (3 mm in diameter) distributed along the nasal–temporal and the superior–inferior axes, as shown in Fig. 4A. The results of analysis of seven eyes (six animals) are given in Table 1. The same results are shown in Fig. 4B, with the exception of that for animal 88105, for which precise eccentricity information was not available. There was no statistically significant variation in the L:M ratio with eccentricity of as much as 9 mm (~45°). On the other hand, the L:M pigment mRNA ratio in the nasal retina was consistently lower than in other regions, particularly the temporal, in 10 eyes (9 animals) (Tables 1 and 2; Fig. 4C). The nasal/temporal ratio was approximately 0.69 ± 0.14 ($p = 0.022$; two-tailed t test; $N = 10$ eyes; 9 subjects).

C. Correspondence to Physiological Measurements

In 11 instances the retinal pieces that we used to examine variation in the ratio of L:M cone pigment mRNA were derived from physiological experiments designed to measure the relative synaptic strength of L and M cone input to second-order interneurones, the H1 horizontal cells.[25] One finding of that study was that L:M cone input gain varies greatly as a function of retinal location and across individual retinas, similar to the L-versus-M mRNA re-

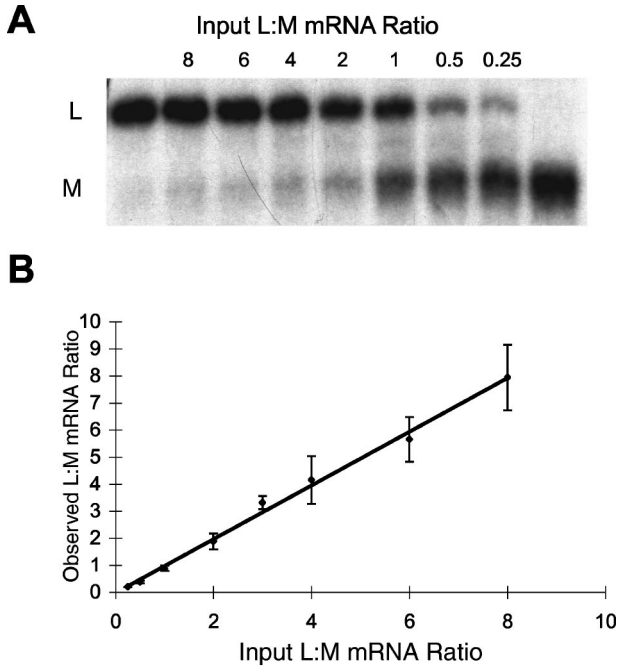

Fig. 2. Standard curve for quantification of the L:M mRNA ratio. A, Phosphor image of a single-strand conformation gel, showing the separation of L from M pigment cDNA strands. Known ratios (indicated above the lanes as input L:M mRNA ratios) of purified L and M pigment cDNA were generated and were used as templates in the competitive PCR amplification reaction by use of primers 148 (end labeled ^{32}P) and 78 (see Fig. 1B), the sequence of which matches both L and M templates. The L- and M-derived PCR products were resolved by electrophoresis, as described in Section 2. Band densities were determined with a phophorimager. B, Plot of input and observed [±1 standard deviation (SD)] L:M cDNA ratios.

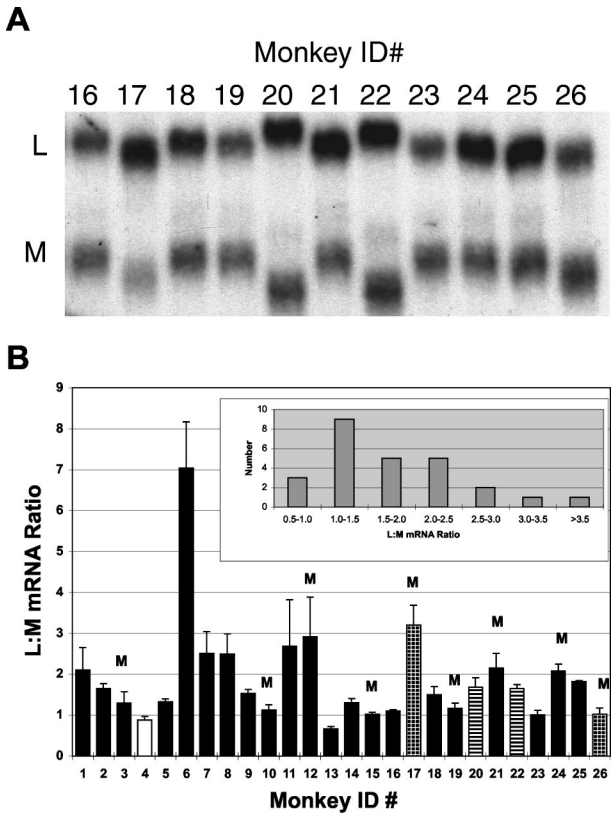

Fig. 3. L:M mRNA ratios in whole monkey retinas. A, Phosphor image of a single-strand conformation gel of PCR-amplified segments. RNA was isolated from whole monkey retinas, reverse transcribed into cDNA, and used as a template to amplify the segment shown in Fig. 1B. Monkey identification (ID) numbers are indicated above the lanes and correspond to those in B. Monkeys 17 and 26 are *P. anubis* males, 20 and 22 are *M. fascicularis* females, and the rest are *M. nemestrina* males (monkeys 19, 21, 24) and females (monkeys 16, 18, 23, 25). Differences in mobility of the bands from different species reflect sequence differences. B, L:M mRNA ratios (±1 SD) estimated from band densities shown in A. Monkey ID numbers are shown on the abscissa. Males are indicated by the label M above the bars. Black bars, *M. nemestrina*; white bar, *M. mulatta*; striped bars, *M. fascicularis*; black bars with white squares, *P. anubis*. Inset, frequency distribution of the L:M mRNA ratios.

sults presented here. Moreover, the electrophysiological results support the lack of variation in the L:M pigment mRNA ratio with eccentricity and the relatively lower ratios in nasal regions of the retinas. A simple hypothesis is that both the molecular genetic data and the physiological data are determined by the relative numbers of L and M cones in the sample tissue. To determine whether the two independent measures were correlated, we plotted the proportion L cone contribution for mRNA and for H1 cells determined for the same piece of retinal tissue (Fig. 5). The two measures were highly correlated (correction coefficient, 0.85), consistent with the hypothesis that the relative L and M cone numbers underlie the variability seen in both mRNA data and physiological indicators of the L:M cone ratio.[22]

4. DISCUSSION

Previously we developed a rapid methodology that is based on PCR–single-strand conformation polymorphism

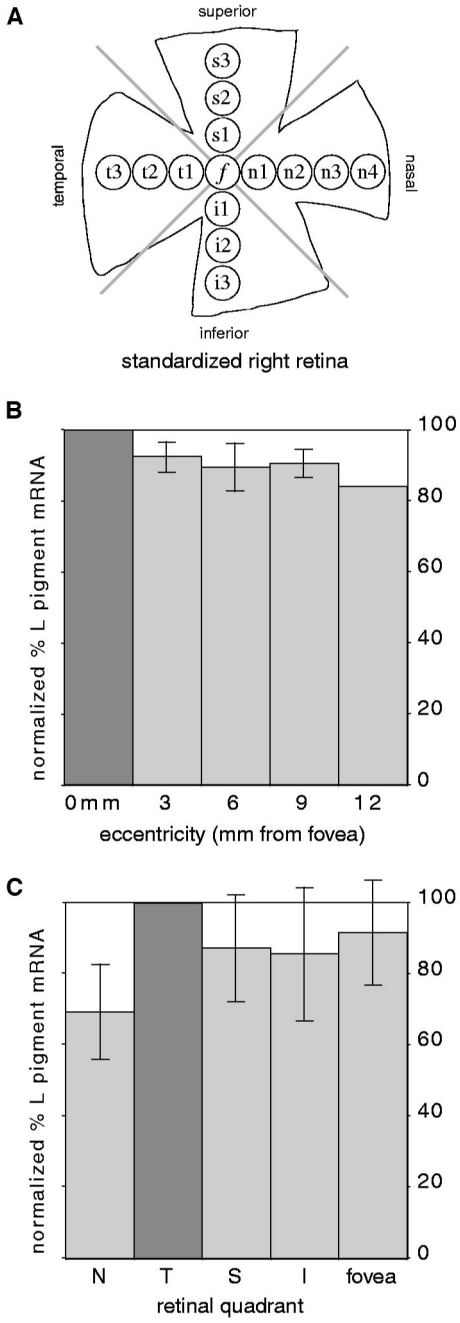

Fig. 4. Relation of retinal location to L:M mRNA ratio. A, Schematic retina illustrates how retinal tissue was sampled. We sampled circular punches ~3 mm in diameter along the horizontal and vertical meridians, noting retinal eccentricity, in millimeters from the fovea. Diagonal lines delineate retinal quadrants, as indicated. B, Histogram of percentage of L pigment mRNA normalized to foveal values as a function of eccentricity. Data are for the retinas listed in Table 1, with the exception of monkey 88105, for which precise eccentricity information was not available. No significant change in the L:M pigment mRNA ratio as a function of retinal eccentricity out to 9 mm was present. Values are means from five retinas; error bars indicate ±1 SD. Data at 12 mm are based on one retina. C, Histogram of percentage of L pigment mRNA by retinal quadrant (N, nasal; T, temporal; S, superior; I, inferior) normalized to the temporal quadrant; error bars indicate ±1 SD. The temporal quadrant shows the highest L pigment mRNA level; nasal retina shows a consistently large reduction of ~30% relative to the temporal quadrant ($p = 0.022$; two-tailed t test). Data are for the 10 eyes (9 animals) listed in Tables 1 and 2.

Table 1. L:M Pigment mRNA Ratios in Monkey Retinal Sections in 3-mm Punches along the Nasal–Temporal and Superior–Inferior Axes[a]

	Monkey Identification Number						
						88105	
Properties	89251	96111	95265	87264	91048	Left Eye	Right Eye
Species	*M. nemestrina*	*M. nemestrina*	*M. nemestrina*	*M. nemestrina*	*M. fascicularis*	*M. fascicularis*	*M. fascicularis*
Sex	Female	Female	Male	Female	Female	Female	Female
Section							
Superior-1	2.23 ± 0.02	1.58 ± 0.26	1.13 ± 0.16	2.66±0.12	1.73 ± 0.36	1.98 ± 0.22	ND
Superior-2	1.98 ± 0.15	1.03 ± 0.21	1.33 ± 0.32	3.35 ± 0.19	1.41 ± 0.14	2.33 ± 0.25	ND
Superior-3	2.00 ± 0.63	0.95 ± 0.26	1.78 ± 0.31	ND	2.24 ± 0.12	ND	ND
Inferior-1	1.98 ± 0.16	0.97 ± 0.21	1.56 ± 0.25	2.41 ± 0.12	1.63 ± 0.14	2.35 ± 0.17	ND
Inferior-2	1.95 ± 0.38	0.71 ± 0.23	1.57 ± 0.21	2.56 ± 0.06	1.39 ± 0.14	2.32 ± 0.10	ND
Inferior-3	1.76 ± 0.02	ND	ND	ND	1.54 ± 0.72	ND	ND
Nasal-1	1.29 ± 0.11	0.86 ± 0.04	1.57 ± 0.20	2.77 ± 0.55	1.42 ± 0.14	1.52 ± 0.11	2.26 ± 0.13
Nasal-2	1.17 ± 0.01	0.63 ± 0.02	1.16 ± 0.12	2.21 ± 0.25	1.34 ± 0.16	1.52 ± 0.27	2.21 ± 0.19
Nasal-3	1.22 ± 0.12	0.76 ± 0.11	1.09 ± 0.03	1.92 ± 0.10	ND	1.12 ± 0.18	3.21 ± 0.11
Nasal-4	ND	ND	1.30 ± 0.12	ND	ND	ND	3.07 ± 0.32
Temporal-1	2.65 ± 0.08	ND	2.15 ± 0.26	3.13 ± 0.22	1.81 ± 0.19	2.16 ± 0.10	2.90 ± 0.18
Temporal-2	2.48 ± 0.45	ND	1.98 ± 0.23	3.39 ± 0.34	1.77 ± 0.21	2.31 ± 0.22	3.02 ± 0.17
Temporal-3	2.56 ± 0.33	1.48 ± 0.23	ND	ND	1.61 ± 0.09	ND	3.18 ± 0.15
Fovea	2.62 ± 0.20	1.23 ± 0.27	2.04 ± 0.34	3.13 ± 0.20	1.81 ± 0.12	1.55 ± 0.09	2.27 ± 0.14
Remainder	2.49 ± 0.08	0.93 ± 0.25	1.35 ± 0.34	2.85 ± 0.33	1.52 ± 0.09	ND	ND

[a] Each value is the average of three independent determinations (±1 SD). ND, not determined.

Table 2. L:M Pigment mRNA Ratios in Patches from the Superior, Inferior, Temporal, and Nasal Regions[a,b]

	Monkey Identification Number		
	*J*91076	*J*9026	88296
Sex	Male	Male	Female
Axis			
L-superior	1.15 ± 0.24	2.20 ± 0.24	0.86 ± 0.09
L-temporal	1.71 ± 0.04	2.38 ± 0.45	1.11 ± 0.07
L-temporal	1.85 ± 0.08	2.78 ± 0.27	ND
L-nasal	1.32 ± 0.01	1.96 ± 0.05	0.70 ± 0.06
L-nasal	ND	2.74 ± 0.16	ND
L-inferior	ND	ND	0.72 ± 0.09
Remainder	ND	2.28 ± 0.68	ND

[a] Each value is the average of three independent determinations (±1 SD).
[b] Species, *M. nemestrina*.

to determine the L:M pigment mRNA ratios in whole human retinas. This ratio ranged from 1 to 10, with a mode of 4, in Caucasian males. In the experiments reported here we applied this method to estimate the mRNA ratio in whole retinas of male and female monkeys as well as in various regions of the retina and compared these with the L:M cone ratios obtained by physiological means. As was found in human retinas,[13] the majority of whole monkey retinas had more L than M pigment mRNA. The average L:M mRNA ratio among monkeys (the majority of monkeys were *M. nemestrina*) ranged from 0.66 to 7, with a mean of approximately 1.6 ± 0.6. This ratio is significantly different from ratios in humans, raising the possibility that large differences in the ratio may exist among other primate species. The mRNA ratios correlated well with the L:M cone ratios inferred from electrophysiological recordings of cone signal gain in H1 horizontal cell interneurons in the same retinas.[25] Thus the correlation between L:M mRNA ratios and physiological cone gain ratios supports the conclusion that both measures reflect the ratio of L:M cones in the retina. The observation that the L:M mRNA ratios in monkeys is smaller than observed among humans is consistent with previous findings by microspectrophotometry[18,19] and ERG.[22] There was more variation in the L:M ratio among the subjects in this study than was observed among the 42 male subjects (*M. mulatta* and *M. fascicularis*) studied by ERG,[22] perhaps reflecting differences in the degree of genetic heterogeneity.

We observed no change in the L:M mRNA ratio in monkey retinas with eccentricities of as much as 9 mm (~45°). This result is in contrast to what was observed in 25 male human retinas, in which the L:M mRNA ratio was similar at central and mid periphery but increased substantially (by approximately a factor of 2) in far-peripheral regions (41° of eccentricity from the fovea).[14] Interestingly, we observed an approximately 30% higher L:M pigment mRNA ratio in temporal than in nasal retina, indicating the presence of a temporal–nasal gradient. The significance of this gradient and how it is established would be interesting issues to explore. In contrast, no significant difference in the L:M mRNA ratio was observed between nasal and temporal patches of two male human retinas.[14]

It would be important to explore the mechanisms that determine the L:M cone ratio and whether the observed interindividual differences constitute a heritable trait. A mechanism that would allow the mutually exclusive expression of the X-chromosome-linked L and M pigment

genes in their respective photoreceptors has been proposed.[13,29,30] In this mechanism a locus control region (LCR), located between 3.1 and 3.7 kbases 5′ of the L pigment gene, was shown to be required for cone-photoreceptor-specific expression of both L and M pigments. Thus, if the LCR forms a stable transcriptionally active complex with the L opsin gene promoter, an L cone will result. If the LCR forms a stable complex with the proximal M pigment gene promoter, an M cone will be formed instead. Thus the choice to form an L or an M cone is predicted to be stochastic. LCR-driven mutually exclusive expression of reporter genes in transgenic mice has been demonstrated.[31]

The observed dominance of L over M cones in the majority of primate retinas may be explained in part, by the proximity of the L pigment gene to the LCR. Distance-dependent gene expression by LCR's was demonstrated for the β-globin gene locus[32] and for the L–M visual pigment gene array.[13,14,33] In addition to proximity to the LCR, differences in the sequences of the LCR, of the L and M pigment gene promoters, or of both may play a role in determining the ratio of L:M cones. This hypothesis predicts that interindividual differences in the L:M cone ratio are inherited in an X-chromosome-linked fashion. Evidence for a difference in promoter sequence that favors the M pigment gene promoter was suggested by *in vitro* promoter analysis.[34] Thus individual differences in the sequences of the promoter or LCR that influence the affinity of the LCR to the promoter may partly underlie individual differences in L:M cone ratio. Support for this hypothesis comes also from family studies of transmission of the L:M ratio, as estimated by photometric matches.[6,35]

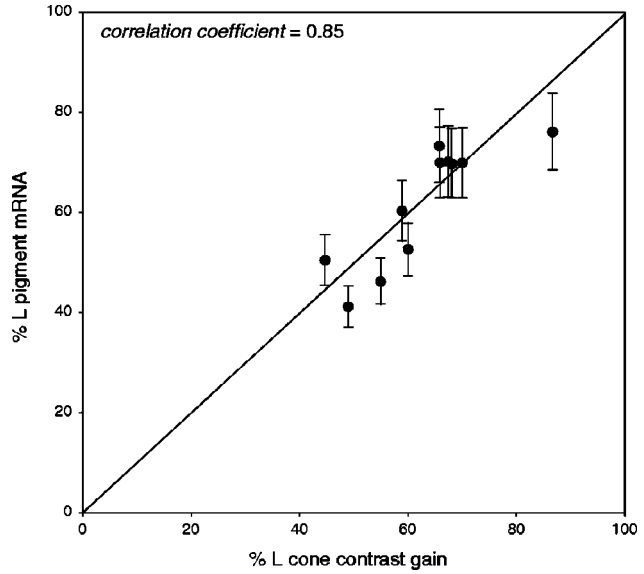

Fig. 5. Plot of percentage of L cone contrast gain for H1 horizontal cells against percentage of L pigment mRNA level for the same piece of retinal tissue. The two data sets are highly correlated (correlation coefficient $r = 0.85$; two-tailed t test; $P = 0.05$). A line with a slope of 1 is drawn through the data points. The values for the mRNA are the means of three measurements; error bars indicate ± 1 SD. Values for the H1 cell contrast gains are taken from cells located within a 3-mm-tissue punch that was used for the mRNA analysis. Multiple measurements of M and L cone contrast gain were made on each H1 cell, and the data points are mean values. SD's were small, ranging from 0.5% to 1.5% of the mean, and are not shown.

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