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Characterization of a novel form of X-linked incomplete achromatopsia

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Abstract

X-linked incomplete achromatopsia (XIA), also called blue-cone monochromacy (BCM), is a rare cone disorder that most commonly results either from one of two conditions.. The first condition is a deletion of the locus control region (LCR) which is a critical DNA element that lies upstream of the L and M photopigment gene array on the X-chromosome and is necessary for expression of the photopigment genes. The second condition is an inactivating point mutation within the coding sequence of the remaining photopigment gene in an array from which all but one gene has been deleted. Many previous studies have concluded that affected individuals either have only rods and S-cones (Blackwell & Blackwell, 1957, 1961; Daw & Enoch, 1973; Hess et al., 1989) or have rods, S-cones, and another cone type that contains the rod pigment (Pokorny et al., 1970; Alpern et al., 1971). However, Smith et al. (1983) described individuals with XIA who had residual L-cone function. Here we report results for a subject with XIA who appears to have residual M-cone function. Genetic analysis revealed that he had apparently normal genes for M-cone photopigment thus leaving open the possibility that he has a contribution to vision based on expression of these genes at a very low level.

Keywords: Blue-cone monochromacy, Cone disorder, Color blindness, Monochromat, Genetics

Introduction

X-linked incomplete achromatopsia, or blue-cone monochromacy, is a class of cone dysfunction characterized by severe color-vision deficits. The characteristics of visual function in XIA are variable in severity and include reduced visual acuity, nystagmus, and photophobia. Measurements of luminosity in affected individuals typically reveal a scotopic function at dim background levels. Increment threshold spectral-sensitivity measurements on high-intensity backgrounds usually reveal the presence of S-cones. Fundus appearance in XIA may be normal or may show age-dependent macular abnormalities. The visual anomalies seen in XIA have been proposed to result from a complete loss of L- and M-cone function (Blackwell & Blackwell, 1957, 1961; Daw & Enoch, 1973; Hess et al., 1989). Residual color vision in these individuals must then be attributed to S-cone/rod interactions or from weak spectral information supplied by retinal inhomogeneities or differences in optical density of the remaining photopigments (He & Shevell, 1995; Crognale et al., 1999) or from the presence of a second cone type containing the rod pigment (Pokorny et al., 1970; Alpern et al., 1971). However, Smith et al. (1983) reported a family with XIA wherein there was evidence for residual L-cone function in four of five affected males; however, there have been no reports of affected individuals with residual M-cone function.

Genetically, XIA cases fall into one of two categories (Nathans et al., 1989, 1993); either there is a disruption of the locus control region (LCR) which is a DNA regulatory element that is contained within a 600-bp DNA segment that lies between 3.1 and 3.7 kilobase pairs upstream of the tandemly arrayed L and M photopigment genes on the X-chromosome, or the X-chromosome visual pigment gene(s) contain inactivating mutations (e.g. Crognale et al., 2001; Nolan et al., 2003).

Here we report the results of experiments to characterize visual function in a subject (MF) with XIA who exhibited many of the typical defining characteristics of XIA, with the exception that he appeared to have residual M-cone function. Genetic analysis of his X-linked photopigment gene array was performed to determine whether it contained any of the previously identified mutations that cause blue-cone monochromacy, and to determine whether he had an intact M pigment gene, which would be a prerequisite for residual M-cone function.

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Materials and methods

Subjects were tested using psychophysics, electrophysiology, and molecular genetics techniques. All experiments involving human subjects were conducted in accordance with the declaration of Helsinki, and were approved by institutional review boards at the University of Nevada and the Medical College of Wisconsin.

Electrophysiology

Electroretinograms (ERGs) were recorded using several systems. Photopic and scotopic flash ERGs were run on an Espion System (Díagnosys, Littleton, MA) using DTL fiber electrodes and the standard ISCEV protocol. Flicker ERGs at 30 Hz were recorded using two systems. One was a three-channel Maxwellian view optical system combined with amplifiers (Grass Telefactor, West Warwick, RI) and a National Instruments (Austin, TX) IO board in a PC and the other was the Espion system employing a handheld ganzfeld strobe.

Visual evoked potentials (VEPs) were recorded using a Grass amplifier, and a National Instruments board in a PC. The active electrode was placed at Oz, and reference and ground electrodes were placed on the earlobes. VEP stimuli were generated using the Cambridge graphics board and displayed on a Sony monitor. Patterns were 0.5 cycles/deg (cpd) horizontal sinusoidal gratings presented in an onset–offset mode (200 ms ON, 800 ms OFF) viewed at 57 cm and subtending 21 deg. The chromaticities of the patterns were chosen to selectively activate the L-M and S-(L+M) chromatic pathways (Rabin et al., 1994) and were modulated around white (CIE $X = 0.290$, $Y = 0.304$) at a luminance of 42 cd/m^2. The waveform component peaks and troughs were picked by visual inspection.

Psychophysics

A battery of psychophysical tests of color vision were administered that included the Ishihara plate test (38-plate edition), the Neitz Test of Color Vision, the FM-100, the D-15, the DSat D-15, Berson's test, the Cambridge Colour Test, and a Rayleigh match using an Oculus anomaloscope. All plate and arrangement tests were performed monocularly under standard illumination. The Cambridge Colour test was conducted monocularly and in a darkened room. To accommodate our subjects with low visual acuity from XIA, viewing distance was decreased to 142 cm so that the gap in the "C" subtended 2.0 deg.

In addition to standard color-vision tests, increment threshold spectral sensitivities were obtained using a three-channel Maxwellian view optical system described previously (Haegerstrom-Portnoy & Verdon, 1991, 1999). Estimates of individual luminosity were obtained using a minimum-motion paradigm (Cavanagh et al., 1987) generated with a Cambridge VSG graphics board, using procedures detailed in Webster and Mollon (1997). The patterns were 1 cycle/deg horizontal gratings drifting at 1 Hz, and were varied within the LM and S planes of the MacLeod-Boynton-Derrington-Krauskopf-Lennie (MBDKL) three-dimensional color space around a fixed mean (30 cd/m^2 and chromaticity of Illuminant C). Chromatic contrasts were the maximum available on the monitor within each plane. Luminance contrast in the gratings was varied with a staircase to estimate the motion null.

Limited numbers of measurements were made following a "rod-bleach" that attempted to minimize rod contribution. These measurements were made between 3 and 6 min after a 5-s exposure to bright, white light (4×10^7 scotopic trolands) produced from a three-channel, Maxwellian view system with a field that subtended 53 deg.

Genetic analyses

The relative numbers of L and M photopigment genes on the X-chromosome of subject MF and his affected brother were estimated using two real-time quantitative polymerase chain reaction (PCR) assays that were described previously (Neitz & Neitz, 2001). In the assay designed to determine the L/M gene ratio, the forward primer corresponded to sequences within exon 5, and the reverse primer spanned the junction between exon 5 and intron 6. Both primers hybridized to sequences shared by L and M genes. Two probes were in each reaction—one specifically hybridized to L genes and was tagged with the fluorescent dye 6-FAM, the other was specific for M genes and was tagged with the fluorescent dye JOE. The primer and probe sequences and the reaction conditions were reported previously (Neitz & Neitz, 2001). The fluorescence intensity from each probe increases as a function of PCR cycle number in direct proportion to the amount of PCR product made from the sequences to which the probe hybridizes. The relative increase in fluorescence intensity was measured as a function of time for each probe, and was used to estimate the L/M gene ratio. In the assay to estimate the ratio of first/downstream genes, the forward primer corresponded to sequences just upstream of exon 1, and the reverse primer corresponded to sequences within exon 1. The primer sequences are shared by L and M genes. Two probes were used in each reaction; they were shown empirically to distinguish first from downstream genes (Neitz & Neitz, 2001). One probe was specific for the sequence 5′ to exon 1 in the first gene in the array and was tagged with the fluorescent dye 6-FAM, and the other was specific for downstream genes and was tagged with the fluorescent dye JOE. The relative fluorescence intensity from each probe was measured as a function of time and used to estimate the first/downstream gene ratio. The primer and probe sequences and reaction conditions were described in detail previously (Neitz & Neitz, 2001). The assays were calibrated using standard curves generated by mixing standard DNA samples of known sequence in specific ratios, as described previously (Neitz & Neitz, 2001). In addition, control genomic DNA samples of known L/M gene ratio and first/downstream gene ratio were run in parallel with the assays on the experimental subjects. Each sample (control and experimental) was assayed in quadruplicate for L/M and first/downstream gene ratios; the gene ratios reported are the average of the quadruplicate reactions. It was shown previously that the 95% confidence interval is ± 4% for each gene ratio estimate when quadruplicates are averaged. The 4% error represents the total error in the measurement, including errors in sample loading, fluctuations in the reagents, and the long-term stability of the instrument.

The L and M photopigment genes for each subject were separately and specifically amplified, and the DNA sequence was determined for exons 2, 3, and 4 of the L and M genes as described previously (Carroll et al., 2000). Long-distance PCR and sequence analysis was done as previously described (Kainz et al., 1998; Hayashi et al., 1999) in order to determine the identity of the first and last gene in each array as encoding an L or an M pigment. A segment of DNA encompassing the LCR was amplified using the reverse primer 5′CTGGGAACCAAACTCAGACGCCC, and the forward primer 5′GCCCCCACAGGTGCTGAGTGACT. These primers flank the 600-bp segment containing the LCR identified by Nathans et al. (1989) as critical for L and M gene expression.

The LCR fragment was amplified from MF's genomic DNA using the XL-PCR kit according to the manufacturer's recommendations for the concentration of all reaction components, and thermal cycling parameters. The annealing/extension temperature used was 68°C.

Results

Subject MF was a 23-year-old male with a family history of X-linked achromatopsia (described below). Ophthalmic exam on subject MF revealed several relevant findings. His best-corrected visual acuity was 20/200 (Bailey-Lovie chart). He had rather extreme myopia (*ca.* 19 diopters) in part due to an unusually long axial eye length of 30.5 mm. Examination of the fundus did not reveal any obvious anomalies other than a somewhat darkened appearance overall despite light skin pigmentation. MF had normal pupillary reflexes and intraocular pressures.

Electrophysiology

Electrophysiological results will be described only briefly here and will be described more completely in a future publication. ERG recordings revealed a slightly diminished scotopic response and a photopic response that was entirely absent. We were also unable to see any evidence of a flicker ERG at 30 Hz using a standard handheld ganzfeld flash unit (the Colorburst) from Espion. Further tests using a three-channel Maxwellian view system to stimulate with a 30-Hz square wave similarly yielded no response, even at retinal illumination levels of about 1×10^6 tds. Casual observations during ERG testing revealed that MF could reasonably suppress his nystagmus and control his light aversion.

We also employed the chromatic pattern onset VEP to evaluate the integrity of the chromatic pathways (Crognale et al., 1993). We were unable to record reliable responses to chromatic stimuli that isolated the L-M chromatic pathway in subject MF. MF did produce a small but reliable S-(L+M) pathway response that was greatly delayed (*ca.* 210–220 ms) compared to color-normal subjects (*ca.* 120–140 ms). The achromatic (luminance) response from MF was reduced in amplitude but had normal latency. The VEP results suggest the presence of weak S-(L+M) or "blue–yellow" pathway response but no evidence for any L-M or "red–green" pathway function.

Psychophysics

Fig. 1 shows the results of the Cambridge Colour Test (the trivector test) and plots discrimination from the center white point along three different confusion axes (P- protan, D- deutan, and T-tritan) plotted in CIE U′V′ coordinates. Results from MF are shown in the top two panels. Results from a control subject (JH) are shown in the lower panels for comparison. Threshold values along the three axes are indicated. According to the design of the Cambridge Colour Test, values in excess of 100 are considered anomalous.

The thresholds for MF without a rod bleach (left) indicate that his color discrimination is extremely poor along the protan and deutan directions scoring well outside the normal range. His thresholds along the tritan axis are better but are still about two times the normal limit. After a rod bleach (right), his thresholds increase along all axes but particularly along the tritan axis wherein it is doubled. This loss of discrimination implicates rod contribution to tritan discrimination in the unbleached condition.

The bottom panel displays the thresholds for our control subject JH. As expected, the thresholds along each of the protan, deutan, and tritan directions are very close to the average chromaticity indicating good color discrimination. The bottom right panel shows the performance for the control subject after a rod bleach. As expected, there were no significant losses observed for the control subject after a rod bleach suggesting that rod contribution was not responsible for the good color discrimination observed without a bleach.

Table 1 summarizes the results of color-vision tests performed by subject MF. Subject MF failed most of these tests. However, MF passed the tritan plates of the Neitz Test of Color Vision and the Berson test. He made predominantly protan errors on the D-15 and the desaturated D-15. He could also set a broad Rayleigh match on the Oculus anomaloscope that indicated a protan defect, with a brightness-match slope that was somewhat steeper than a typical protan's, although still far from a complete achromat. MF had no discernible axis on the FM-100 test.

Fig. 2 shows the results of minimum-motion settings for stimuli modulated within the S and LM planes of the MBDKL color space. Settings are plotted as elevation relative to the isoluminant plane calculated from the Smith and Pokorny fundamentals and the Judd modified CIE luminosity function. Within the S plane, subject MF made minimum-motion settings intermediate between the scotopic prediction and the photopic, L-cone, and M-cone predictions. There was a small shift away from the scotopic axis after a rod bleach. Within the LM plane, subject MF made settings between the photopic prediction and the predictions for the M-cones and rods. Again there was a small shift away from the rod prediction after the bleach. The control subject made settings close to the photopic prediction within the LM and S planes and for both bleached and unbleached conditions, as expected. Taken together

Table 1. *Color-vision test results*

Test	Results
Neitz Test of Color Vision (WPS)	Passed tritan plates, failed all others
Farnsworth Lantern Test	Failed
D-15	Failed, mostly protan errors
Lanthony's Desat D-15	Failed, mostly protan errors
FM-100	Total Error Score-260, low discrimination; Vingry's: angle = 36.6, C = 2.93, S = 1.59, unresolved
Ishihara 38 Plate Test	Failed; missed plates 2–17, failed to recognize 3 of 4 "positive" plates (18–21), failed diagnostic plates (22–25)
Oculus Anomaloscope	Protan defect, AQ = 0.27–0.01; match endpoints/brightness = 59.5/11.1–72.5/1.6.
Berson's Test	Passed

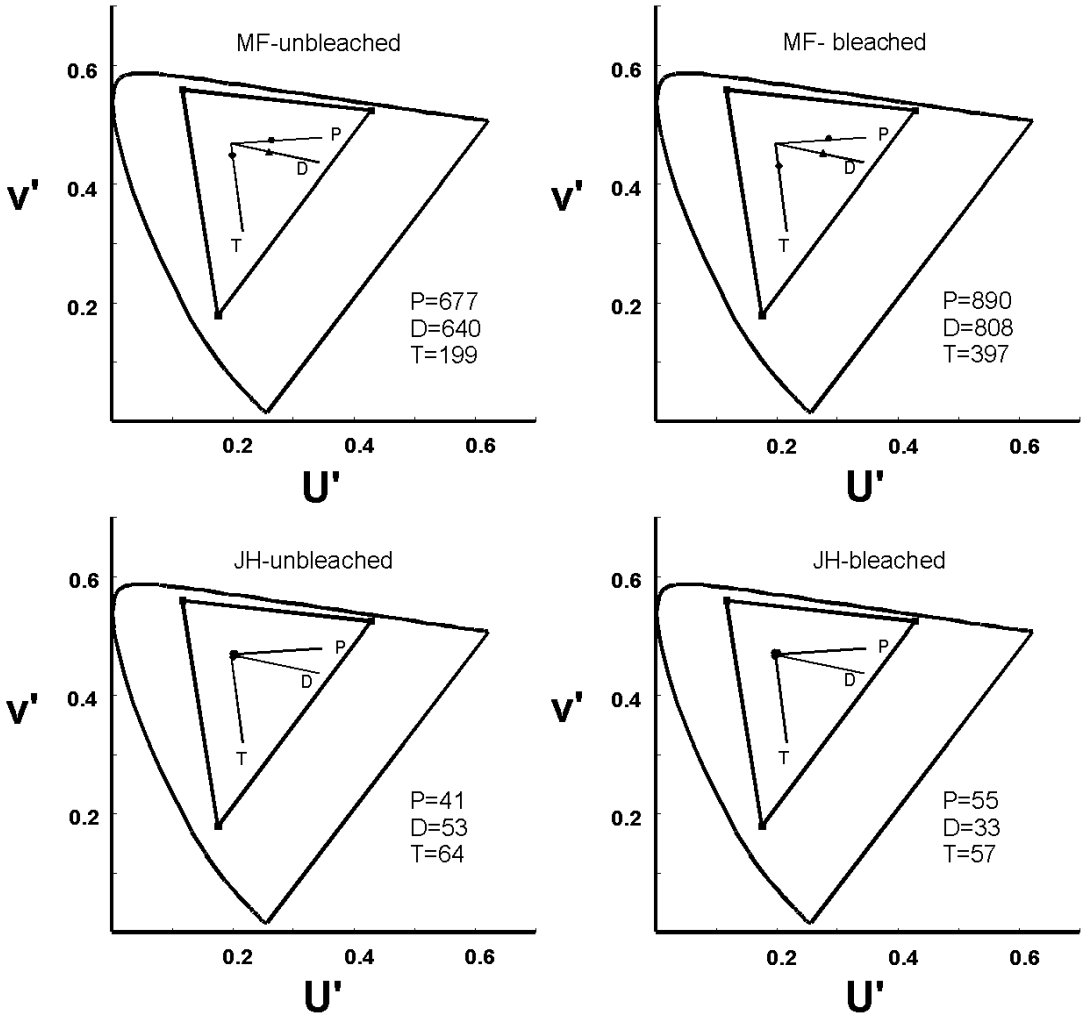

Fig. 1. Results from the Cambridge Colour Test for a subject with X-linked incomplete achromatopsia (MF-upper panels) and a control subject (JH-lower panels). Thresholds are plotted from white in CIE 1975 U.C.S. coordinates. Protan, deutan, and tritan confusion axes and threshold values are indicated by P, D, and T, respectively. The panels on the left show monocular thresholds without a rod bleach while those on the right indicate thresholds after a rod bleach.

these results are strong evidence for residual M- or M- and L-cone contribution to luminosity in our subject with XIA.

Fig. 3 shows the results of increment threshold spectral sensitivities obtained from subject MF measured on a dim yellow background (top), a bright yellow background (middle), and a blue background (bottom). The data obtained on a dim yellow background reveal a rod spectral sensitivity. The curve drawn through the data points is V_λ' and provides a reasonable fit. The spectral sensitivity obtained on a bright yellow background reveals the presence of at least two spectral mechanisms. The curves drawn through the points are those for the S-cone and M-cone spectral sensitivities and provide an excellent fit to the data. The spectral sensitivity obtained on a blue background is shown in the lower panel and are well fit to the M-cone spectral-sensitivity template. The absolute sensitivities shown in the middle and lower panels are reduced relative to those of normal and anomalous subjects measured on the same apparatus (data not shown). These increment threshold spectral-sensitivity data provide strong evidence for the contribution of rods, S-cones, and M-cones to behavioral sensitivity.

The pedigree for MF's family is shown in Fig. 4. Five or six males in the family are reported to be affected, MF and his brother, his maternal great-grandfather, and two or three cousins (the status of one cousin is currently not clear). We have confirmed the affected status of MF's brother, but have not yet examined the other family members reported to be affected. The pedigree shows a typical X-linked pattern of inheritance for this vision disorder and suggests that MF inherited the genes for the condition from a maternal great-grandfather. MF's mother is an obligate carrier as is her twin sister.

Genetic analysis

It has been established that deletion of a 600-bp fragment that contains the locus control region results in blue-cone monochromacy, presumably due to the absence of expression of the X-linked photopigment genes (Nathans et al., 1989). PCR using primers designed to amplify the 600-bp LCR-containing fragment yielded a PCR product of the correct size from subject MF, indicating that it had not been deleted in subject MF or his affected brother.

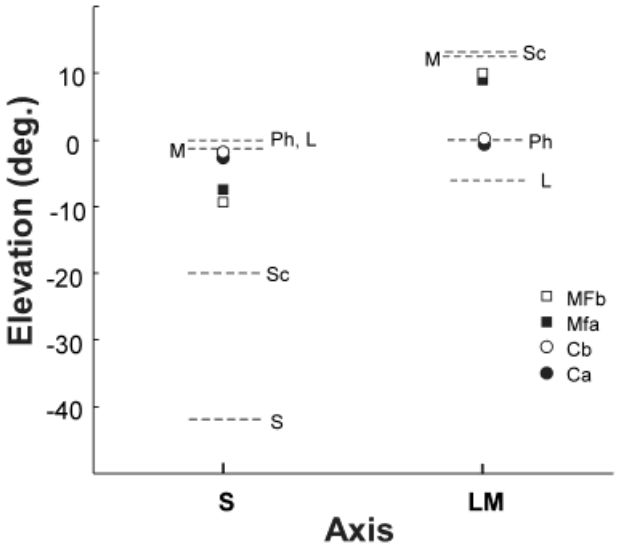

Fig. 2. Isoluminant settings in the S and LM planes of the MBDKL color space for a subject with X-linked incomplete achromatopsia (MF-squares) and a control subject (C-circles) before a rod bleach (open symbols) and after a rod bleach (filled symbols). Predictions for isoluminance settings mediated by rods (Sc), V_λ (Ph), L-cones (L), M-cones (M), and S-cones (S) are shown by the labeled dashed lines.

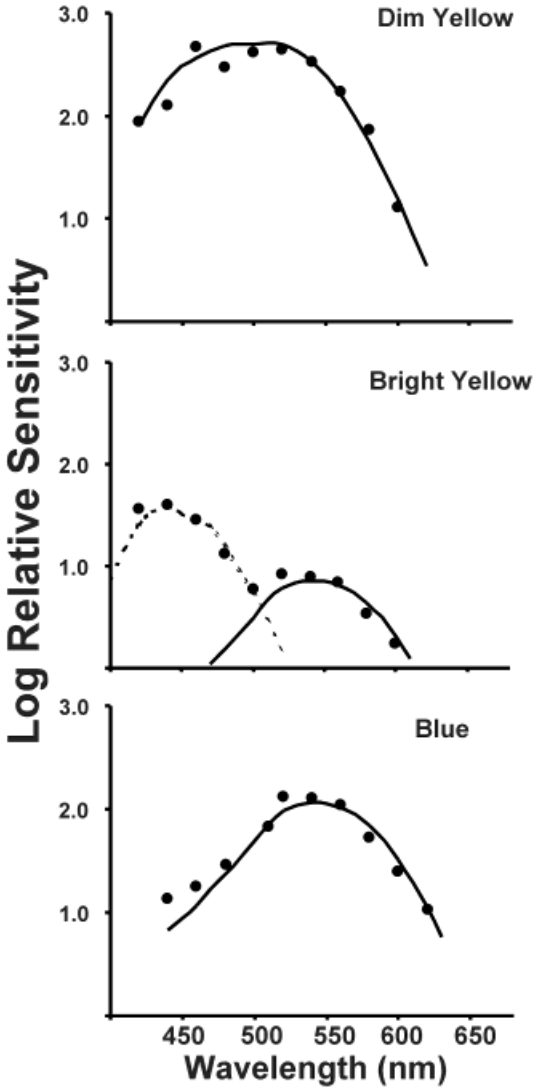

Fig. 3. Increment threshold spectral sensitivities obtained from a subject with X-linked incomplete achromatopsia. The adapting backgrounds for the top, middle, and lower panels were 2.2, 3.2, and 1.8 log photopic tds, respectively. The solid curve in the top panel is V'_λ. The solid curve in the middle and lower panels are M-cone templates. The dashed curve in the middle panel is the S-cone template.

Real-time quantitative PCR was used to estimate the ratio of L/M genes and the ratio of the number of genes in the first position in the array to the number of genes downstream of the first one. This latter provides an estimate of the total number of visual pigment genes on the X-chromosome, as the number of first genes is one. MF was estimated to have 43% L genes, and his brother was estimated to have 48% L genes. MF was estimated to have 76% downstream genes, and his brother was estimated to have 82% downstream genes. Control samples with known numbers and ratios of gene were run on the same plates to insure that the instrument is kept in calibration. Assuming that the brothers have only whole pigment genes these percentages are consistent with the presence of four genes total, two L and two M which would predict measurements of 50% L and 75% downstream genes or five genes total, two L and three M genes which would predict measurements of 40% L and 80% downstream genes. The measurements are about equally consistent with either of these two possibilities. However, they are not consistent with the brothers having one L and two M genes which would predict measurements of 33% L genes and 67% downstream genes

The amino acid differences at positions 277 and 285, both encoded by exon 5, are responsible for producing the spectral difference between pigments of the L-class, which peak near 560 nm, and those of the M-class which peak near 530 nm (Neitz et al., 1991). Sequence analysis of exon 5 of the first and last genes in the X-chromosome array for MF and his brother revealed that both had arrays in which the first gene encoded a pigment of the L-class, and the last gene encoded a pigment of the M-class. The relative order of the genes between the first and last could not be determined.

For both MF and his brother, the L gene(s) were specifically amplified and exons 2, 3, and 4 were sequenced. There are 11 polymorphic amino acid positions specified by these exons (Nathans et al., 1986; Winderickx et al., 1993; Carroll et al., 2000). The particular combination of amino acids specified by the L genes in MF and his brother was threonine 65, isoleucine 111, serine 116, leucine 153, isoleucine 171, alanine 174, valine 178, alanine 180, isoleucine 230, alanine 233, and methionine 236 We have never observed this combination of normal polymorphisms encoded by the more than 300 L- and M-cone pigment genes which we have sequenced in individuals with normal color vision (Neitz & Neitz, unpublished data). Furthermore, the only reported occurrence of this particular combination was in the affected members of a pedigree segregating blue-cone monochromacy in which affected members had an array that contained a single L gene (pedigree H in Nathans et al., 1989). Although the authors did not suspect this combination of normal polymorphisms to be the causative mutation, they did note that it cosegrated with blue-cone monochromacy in 11 of 11 meiosis examined indicating that it is tightly linked to the causative genetic defect.

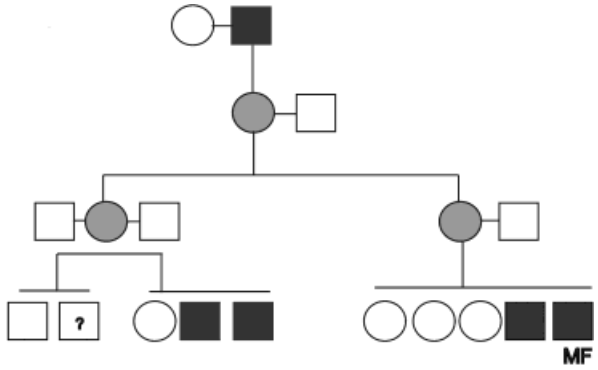

Fig. 4. Inheritance pattern for the family of subject MF, with X-linked incomplete achromatopsia. Squares represent males, and circles represent females. Black squares are affected males, grey circles are obligate carriers, and white symbols are unaffected individuals. The question mark indicates an individual for which the affected status is uncertain.

Discussion and summary

The ERG results demonstrate a clear loss of cone function indicative of a severe cone disorder. VEP recordings suggest that there are gross deficiencies in the chromatic visual pathways even for S-cone stimulation. Delayed or abnormal S-cone responses would be expected because S-cones feed predominantly into color pathways in normal trichromats. When VEPs are recorded to S-cone stimuli in normal trichromats, the resultant waveforms arise from the S-(L+M) color pathway, not some putative S-cone-only pathway. Because L- and M-cones are largely absent in X-linked achromatopsia, normal color pathways are not present and thus abnormal responses should be expected, even with S-cone isolation. The small and delayed response suggests residual chromatic processing most likely arising from opponency between the presumably normal complement of S-cones and residual M-cones or rods.

Psychophysical tests of color vision reveal a general, severe but incomplete color-vision deficit. There remains some tritan discrimination with evidence of rod contribution consistent with the VEP results. Measurements of isoluminance by minimum motion suggest some contribution of longer wavelength cones, most likely M-cones to luminosity. Increment threshold spectral-sensitivity functions fail to reveal any evidence for L-cones but show evidence for rods, residual M-cone, and S-cone contribution to detection.

Molecular genetic characterization of the subject's X-chromosome visual pigment gene array indicates that it contains genes for both L- and M-cone pigments. While very little is understood about the *cis*-acting DNA elements required for X-chromosome visual pigment gene expression, it is known that a 600-bp fragment that contains the LCR is essential for the genes to be expressed and its deletion is associated with blue-cone monochromacy (Nathans et al., 1989, 1993). We demonstrated that this 600-bp LCR-containing fragment is intact in subject MF, and so his condition cannot be attributed to its absence.

When all of the genetic data is taken into consideration, including that MF and his brother share the same extremely rare L pigment gene sequence, and the similarity in the estimates for the L/M and the first/downstream gene ratios, we concluded that they have inherited the same X-linked photopigment gene array from their mother. In addition, the gene ratio estimates support the conclusion that MF and his brother each have an array containing a total of four or five genes (76% and 82% downstream genes, respectively), of which about half are L genes (43% and 48% L genes, respectively). In Fig. 5, we show MF and his brother to have arrays containing two L and two M genes, although they may equally well each have an array with two L and three M genes. However, it is unlikely that they have arrays with only one L and two M genes. Although it is notable that our interpretation means that MF and his brother each have two copies of an L gene with a rare sequence this is not so surprising when one considers that MF was selected for this study because he suffers from a very rare vision disorder.

The rare combination of amino acid polymorphisms specified by the L genes in the XIA subjects in this study was reported previously in one family (pedigree H in Nathans et al., 1989) of blue-cone monochromats, but has never been reported in association with normal color vision. In the affected members of pedigree H, the L gene and the LCR were shown to be normal, yet there was a complete absence of L-cone function. It was concluded that the rare combination of polymorphisms was tightly linked to the genetic defect that caused blue-cone monochromacy in that family. Thus, it seems reasonable to conclude that the same mutation that caused blue-cone monochromacy in pedigree H (Nathans et al., 1989) is present and responsible for the absence of functional L-cones in the XIA subjects in this study. Conclusive evidence that the genotype underlies XIA in this patient will await further experiments

The M pigment genes in the XIA subjects appear to be normal in sequence. One is positioned at the 3′ end of the array, and the

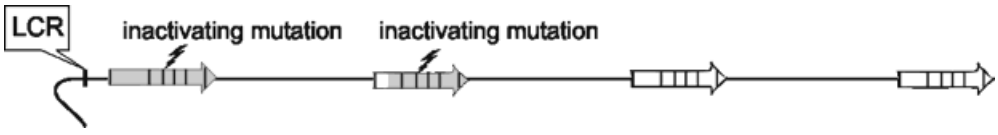

Fig. 5. Deduced structure of the photopigment gene array for subject MF. Shaded arrows represent genes encoding L pigment; white arrows represent genes encoding M pigment. Real-time quantitative PCR yielded an estimate of 43% of the genes in the array encoding L pigment, and 76% of the genes being downstream of the first gene, with 95% confidence intervals of ±4%. The promoters of the downstream genes share the same sequence, which differs from the sequence of the first gene in the array. The box labeled LCR indicates that the 600-bp upstream DNA segment containing the locus control region, that when absent is associated with blue-cone monochromacy (Nathans et al., 1989), is present in this subject. Thunderbolts indicate that the extremely rare amino acid sequence encoded by both L genes is tightly linked to an inactivating mutation that is associated with absence of cone function. The M pigment genes were apparently normal. The array has an L gene at the 5′ end (left) and a M gene at the 3′ end (right) but the relative positions of the genes drawn in the second and third positions are not known.

other is either in the second position or another position between the second and last gene. Previous work has shown that usually only the first two genes in the X-chromosome array are expressed in the retina (Hayashi et al., 1999; Neitz et al., 2002), although exceptions have been reported (Sjoberg et al., 1998). The presence of intact M pigment genes on the X-chromosome of this subject leaves open the possibility that a tiny number of cone photoreceptors express a normal, functional M-cone photopigment, and that this very small number of cones contribute to vision.

Acknowledgments

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