PERGAMON

Vision Research 38 (1998) 3359–3364

Vision Research

Pigment gene expression in protan color vision defects

Shawn D. Balding [a], Stacy A. Sjoberg [a], Jay Neitz [a,*], Maureen Neitz [a]

[a] *Department of Cell Biology and Anatomy and Department of Ophthalmology, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee WI 53226, USA*

Received 10 July 1997; received in revised form 19 November 1997

Abstract

We screened 150 male eye donors and identified four who did not have or express L pigment genes, consistent with each of them having a congenital protan color vision defect. One donor was identified as a protanope because he had and expressed a single X-chromosome photopigment gene that encoded an M pigment. Three were categorized as protanomalous because each expressed significant levels of genes specifying two spectrally different M pigments. The first gene in each of the protanomalous arrays was expressed the most and encoded an M pigment that differed in amino acid sequence from M pigments in color normal men.

Keywords: Color blindness; Gene expression; M cones; Photopigment genes; Protanopia

1. Introduction

Trichromatic color vision requires at least three spectrally different cone photopigments. For color vision to be considered normal, the cone types must be derived from each of three well-separated spectral classes: short, middle, and long wavelength sensitive, abbreviated as S, M, and L respectively.

Inherited red-green color vision defects are common, affecting 8–10% of men in the USA. We are interested in the biological mechanisms that underlie red-green color vision defects. Much has been learned from psychophysical and electrophysiological analysis of color vision and the underlying photopigments, and from molecular genetic analysis of the X-chromosome visual pigment gene arrays. In order to link our understanding of color vision phenotype with genotype, we need information about visual pigment gene expression. Human eye donor tissue can be used to analyze both visual pigment gene expression and the structure of the X-chromosome array in the same person. Array structures that are characteristic of specific color vision defects have been identified [1–4] and this information can be used to infer, with great confidence, the color vision phenotype of eye donors.

The focus of this paper is on visual pigment gene expression in male donors presumed to have had a congenital protan defect. By definition, protan men are missing functional L cones. That loss is virtually always associated with the absence of L pigment genes, producing a gene array structure that is unique to protan defects. This feature of protan men allows us to identify eyes from protan donors. We screened 150 male eye donors for protans by looking for the absence of L pigment gene expression in the retina and the lack of L pigment genes on the X-chromosome. Four such donors were found.

2. Materials and methods

2.1. Subjects and nucleic acid isolation

Blood samples and eyes from male donors were obtained from the Wisconsin Lion's Eye Bank within 5 h of death. A 6 mm punch of retina (centered on the fovea) was taken within 18 h of receiving the eyes, and frozen at −80°C. Nucleic acids were isolated from the tissue as described [5,6].

* Corresponding author. Tel.: +1 414 4568457; fax: +1 414 2668496; e-mail: jneitz@post.its.mcw.edu.

0042-6989/98/$19.00
PII: S0042-6989(97)00440-9

2.2. Assay for L pigment gene expression

We screened retinal nucleic acid samples from 150 male eye donors to look for expression of M and L pigment genes in each retina. messenger RNA (mRNA) was reverse transcribed and the polymerase chain reaction (PCR) was used to amplify a segment of the M and L pigment complementary DNA (cDNA) from retinal tissue. The amplified cDNA segment contained exon 5 of the M and L cDNAs, and the PCR product was digested with the restriction endonuclease Rsa I, which cleaves within exon 5 of L pigment cDNAs, but not M pigment cDNAs.

2.3. Assays to estimate pigment gene ratios and number

Using previously described methods, we screened genomic DNA samples from the 150 donors for M and L pigment genes in the X-chromosome array [7]. Briefly, exon 5 was amplified from L and M genes with intron specific primers, and the PCR products were digested with Rsa I. Donors of interest were also analyzed by a second assay that yields an estimate of the total number of X-chromosome pigment genes in the array [7]. A 137 base-pair fragment upstream from exon 1 was amplified from L and M genes; the PCR products were digested with Sma I and subjected to gel electrophoresis. This digest differentiates the first gene in the array from all downstream genes. The relative intensities of bands corresponding to the first and downstream genes were used to estimate the total number of genes.

2.4. DNA sequence analysis

Genomic DNA from the protan donors was used in long distance PCR to amplify the first gene and the downstream genes separately. The first gene was amplified as described previously [3], producing a PCR product that was approximately 13.5 kb. Downstream genes were amplified with primer 5′TTAGTCAGGCTGGTCGGGAACT which anneals to a region 424 bp upstream of the mRNA start site in downstream genes only, and an intron five specific primer (5′ACGGTATTTTGAGTGGGATCTGCT) which produced a PCR product of approximately 11.1 kb pairs. The conditions used were those described by Neitz et al. [3] for amplification of downstream genes. Exons 2, 3, 4 and 5 were amplified from the long PCR products for direct sequencing using previously described methods [7,8] except that here, the positive strand primers were tagged with the M13 forward primer sequence (M13F), and negative strand primers were tagged with the M13 reverse primer sequence (M13R). Direct sequencing was done with M13 forward and reverse primers using dye-primer chemistry, AmpliTaq FS (Perkin-Elmer-ABI) and an ABI 310 genetic analyzer.

cDNA was used in PCR to selectively amplify exon 2–5 of L or M-pigment cDNA as described in [9]. PCR products were obtained only in the M-specific cDNA amplification, and they were directly sequenced as described by Sjoberg et al. [9]. In addition, a cDNA fragment extending from exon 5 through exon 6 of the M pigment cDNAs was amplified using a positive strand primer (5′tgtaaaacgacggccagtGATGGTCCTGGCATTCTGCTT) specific for M pigment cDNAs and a nonselective negative strand primer (5′caggaaacagctatgaccGCTGCAAGATGCAGTTTCGAA). The lower case letters indicate the M13F and M13R tags respectively. After reverse transcription, cDNAs were amplified in hot start PCR with the XL-PCR kit (Perkin-Elmer). Conditions for thermal cycling were an initial cycle of 95°C for 1.5 min followed by 40 cycles of 94°C for 1.25 min and 70°C for 30 s and then a final extension time of 10 min at 72°C. The PCR products were directly sequenced.

2.5. Restriction digestion assay

The relative levels of expression of the different M pigment cDNAs in individual retinas were estimated by restriction digestion analysis. Restriction site polymorphisms among M pigment cDNAs in individual retinas were identified from the cDNA sequences. PCR amplification of exons 3, 4 and 5, the restriction digestion assays and the estimation of the relative amounts of cDNA in individual bands was done as described previously [8,9]. For the amplification of cDNA fragments used for Fok I digestion analysis, primers identical to those described by Neitz et al. [8] were used (primer numbers 2 and 8) except that they were made with a fluorescein label at the 5′-end.

3. Results

3.1. Identification of protan donors

3.1.1. Screening expressed sequences

There is an Rsa I restriction site in exon 5 of L pigment genes that is absent from M pigment genes. It corresponds to nucleotide differences that encode one of two amino acid polymorphisms that are primarily responsible for the spectral difference between L and M pigments. We used this restriction site polymorphism to screen visual pigment cDNA from retinas of 150 male donors. A segment of the L and M pigment cDNAs containing exon 5 was amplified from each donor, and digested with Rsa I. Donors who express both L and M pigment cDNAs are expected to have cleavage products corresponding to uncut (M pigment cDNA) and cut (L pigment cDNA) PCR product. Donors who had a

protan color vision defect lack L cones, and thus, are not expected to express L pigment genes. Four of the 150 male donors screened were identified by this assay as having had protan defects because only bands from M pigment cDNAs were detected. A total of 138 donors expressed both L and M pigment genes and thus were presumed to have had normal color vision. For eight donors only L pigment cDNA fragments were detected and they were thus diagnosed as having had congenital deutan color vision defects.

3.1.2. Screening genomic DNA

Exon 5 was PCR amplified from L and M pigment genes in genomic DNA from each of the 150 eye donors and digested with Rsa I. Protan men typically lack L pigment genes, and for them, in this assay we expected to see only bands corresponding to M pigment genes. This is what was observed for the four donors who did not express L pigment genes in their retinas (Fig. 1). Finding four protans in 150 male donors is consistent with the fact that about 2% of Caucasian men in the USA have a protan defect.

3.2. Structure of the X-linked pigment gene arrays

For each of the four protan donors, we estimated the number of photopigment genes in the X-chromosome array. One donor, No. 1071, was estimated to have a single X-chromosome visual pigment gene, and thus was identified as an obligate dichromat, a protanope. Two donors (Nos. 901 and 1953) were estimated to have two X-chromosome visual pigment genes, and the fourth donor (No. 734) was estimated to have three genes in the array.

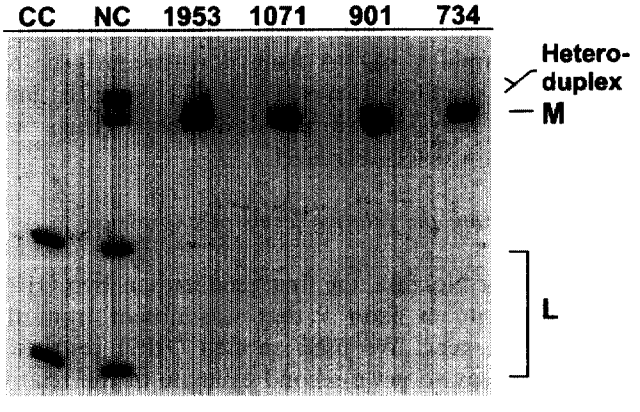

Fig. 1. Rsa I restriction digestion assay of genomic DNA from four protan donors. Exon 5 of the X-linked pigment genes was amplified with fluorescein labeled primers, digested with Rsa I, separated on 12% polyacrylamide, and imaged with a FluorImager (Molecular Dynamics). Lane CC; control for enzyme cutting using genomic DNA from a deuteranope with a single X-linked pigment gene (an L gene), which cuts with Rsa I. Lane NC: control showing banding pattern from an array containing both L and M genes. Lanes No. 1953, 1071, 901, 734: genomic DNA from four male donors who did not express L genes in the retina.

We used long-distance PCR to separately amplify the first gene and the downstream genes in each array, which in turn allowed us to determine the DNA sequence of the first and downstream genes independently. For donor No. 1071, a PCR product was obtained only in the reaction to specifically amplify the first gene. This is consistent with his having a single X-chromosome pigment gene. The sequence of that gene revealed that exon 2 contained sequences corresponding to typical L pigment genes that occupy the first position in the array, while the sequences of exons 3, 4 and 5 in this donor were those most often found in M pigment genes (this is illustrated schematically in Fig. 3).

For the other three donors, the sequences of the downstream gene(s) corresponded to typical M pigment genes found in men with normal color vision. In all three cases, the first gene differed from the downstream gene(s) in the nucleotide sequence, with the first gene having sequences in exons 2, 3 and 4 like those typically found in L genes. The sequence of exon 5 from the first gene in each array was that expected of an M pigment gene. Thus, the M pigments encoded by the first gene versus the downstream genes differed at amino acid positions encoded by exons 2 and 4 which are known to produce spectral shifts [10,11]. We would predict the spectral separation between the encoded M pigments to be sufficient to support protanomalous trichromacy.

3.3. Photopigment gene expression

cDNA amplified from each protan retina was used for direct sequencing. For donor No. 1071, a single cDNA sequence was observed, and it corresponded to the sequence of his only X-chromosome pigment gene. The other three donors showed expression of multiple X-chromosome pigment genes, that is, two nucleotides were evident at positions in the cDNA where differences had been observed between the first gene and the downstream gene(s).

A restriction digestion assay was used to estimate the relative levels of expression of the visual pigment cDNAs in the eyes of the three donors with multiple X-chromosome pigment genes. In exon 4, codon 233 either specifies serine (S233) or alanine (A233), and this difference corresponds to the presence or absence, respectively, of a Dde I restriction site. Amplified visual pigmentation cDNA from each of the four protan retinas was digested with Dde I, the cleavage products were electrophoretically separated and the relative amount of DNA in each band was estimated (Fig. 2A). The relative amount of cDNA derived from expression of the first gene (uncut by Dde I) versus from the downstream gene(s) (cut by Dde I) was calculated, and the proportions of the total were estimated.

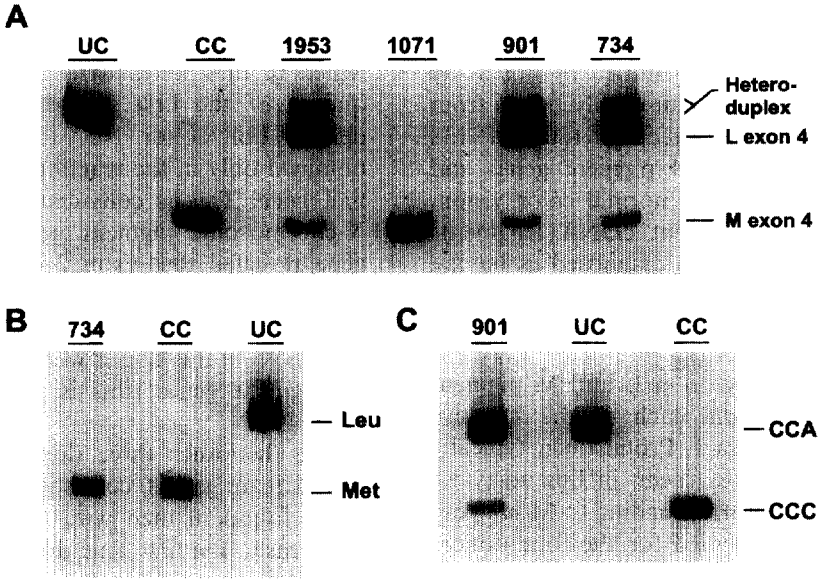

Fig. 2. Restriction digestion assay to determine the relative levels of expression of the genes in individual protan retinas. The appropriate region of the M pigment cDNAs were amplified from protan retinas with fluoresceinated primers, digested with the indicated restriction enzyme, separated on 12% polyacrylamide, and FluorImaged. The amount of DNA in each band was measured, and the relative proportions of the different cDNAs present were calculated as described in [3,7]. (a) Dde I Digests: Lane UC; exon 4 amplified from clone of L pigment cDNA (A233) which does not cut with Dde I. Lane CC; exon 4 amplified from clone of M pigment cDNA (S233) which cuts with Dde I. Lanes No. 1953, 1071, 901, 734; exon 4 amplified from the pigment cDNAs found in the retinas of the protan donors. Heteroduplexes are seen when more than one species of cDNA is present. (b) Lane No. 734; Fok I digest of exon 3 amplified from cDNAs derived from the downstream pigment gene expressed sequences in the retina of donor No. 734. Lane CC; cut control. Lane UC; uncut control. (c) Lane No. 901; Eco O109I digest of exon 5 amplified from the pigment cDNA from the retina of donor # 901. Lane UC; uncut control. Lane CC; cut control.

Donor No. 1071. This donor had a single X-chromosome pigment gene, it encoded S233 and thus the cDNA derived from expression of this gene had the corresponding Dde I cleavage site and only cleaved PCR product was observed (Fig. 2a).

Donors 1953, 901 and 734. For these donors, cDNA derived from expression of the first gene in the array was not expected to be cleaved by the Dde I, but the cDNA from expression of the downstream genes was expected to be cut by this enzyme. Thus, if both the first and downstream genes are expressed, a mixture of cut and uncut PCR product is expected. This is what was observed (Fig. 2A). For each of these three donors, the relative amount of cDNA corresponding to the first gene was much greater than the amount corresponding to the downstream gene(s). For donor No. 901 we estimate that 72% of the cDNA corresponded to the first gene, and 28% to the downstream gene. For donor No. 734, 62% of the cDNA corresponded to the first gene, and 38% corresponded to the downstream genes.

3.4. Are all downstream genes expressed?

Recall that donor No. 734 had two downstream genes. They did not differ at codon 233, so the Dde I digest could not tell us whether one or both of his downstream genes were expressed. Our next experiment was designed to determine the relative levels of expression from the two downstream genes. This was done by, first, specifically amplifying downstream expressed sequences using an exon 4 primer specific for cDNA from the downstream genes. The PCR product, which included exon 3 of the downstream genes, was then digested with the restriction enzyme Fok I. From the genomic DNA sequences, we knew that one of the downstream genes had a Fok I site in exon 3 (i.e. encoded a methionine at codon 153), and the other did not. Amplified cDNA from downstream gene expression was entirely cleaved by Fok I (Fig. 2B) indicating that only one of the downstream genes was expressed at a detectable level.

3.5. Confirming the relative levels of expression

For donor No. 901, the first gene had an Eco O109I restriction site in exon 5 that was absent from the downstream gene. We used an Eco O109I restriction digestion assay to get a second estimate of the relative expression levels of the first and downstream genes in this donor. The results (Fig. 2c) indicate that 73% of the cDNA was derived from the first gene in the array, which was not cleaved by Eco O1091, and 27% of the cDNA was derived from the downstream gene which was cleaved by Eco O109I. This is in good agreement with our values obtained with the Dde I digest of exon 4 from above (72 and 28%, respectively).

4. Discussion

In this study, we investigated the relationship between the structure of the X-chromosome visual pigment gene array and visual pigment gene expression in the retinas from four protan eye donors. Our findings are summarized in Fig. 3. One donor had a single X-chromosome visual pigment gene and was a protanope. His single gene has an exon 2 that is typical of an L pigment gene but the sequences downstream of exon 2 are typical of an M pigment gene. The encoded M pigment differs in amino acid sequence from those usually found in men with normal color vision. Similar gene structures have been observed in living protanopes who were characterized behaviourally [4,11]. We presume that the one gene is expressed in all cones that would have been M and L in a normal eye. If so, then every M/L cone in the eye of this type of protanope expresses a visual pigment that is different from the pigments expressed in normal eyes. The difference, in this case, is not expected to affect the spectral peak of the M pigment but it is possible that there may be other differences in pigment function.

Three of the donors in the present study had and expressed genes encoding two spectrally different M pigments and are presumed to have been protanomalous. In each case, the M pigment gene that was first in the array had a structure that differs from normal M pigments. The pigments encoded by these, upstream-most genes are predicted to have a spectral sensitivity that is shifted toward the long wavelengths compared to normal M pigments. Thus, they embody the characteristics of the classical 'anomalous pigment'. In each case, the 'anomalous pigment gene' that occupied the first position was the one that was most abundantly expressed. Similarly, in men with normal color vision the first gene in the array is expressed at the highest levels. However, normally the first gene is an L pigment gene [12]. The M pigment encoded by the first gene in the protanomalous men differed in amino acid sequence from those usually found in men with normal color vision. The three presumed protanomalous men examined here would be exceptions to our earlier proposal [13] that most anomalous trichromats do not have pigments different than the pigment variants commonly found in normal trichromats. This is in contrast to the growing evidence that supports the idea that deuteranomalous pigments occur and are expressed in normal color vision. For example, the gene structures deduced for deuteranomals [14] are similar to those expressed in individuals presumed to have had normal color vision [9].

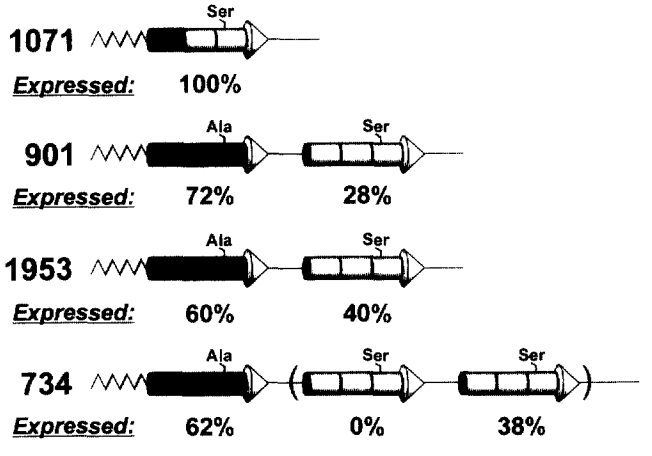

Fig. 3. Structures of the gene arrays and gene expression patterns from four protan donors. Genes are represented by arrows. Segments of the arrows represent respectively (from left) exons 2, 3, 4 and the arrowhead represents exon 5. Light parts of the arrow indicate exons with sequences typical for M genes in color normal men, dark parts indicate exons with sequence more typical of L pigment genes that occur first in the array of color normal men. For donor No. 734, the order of the downstream genes is not known. For each donor, the relative expression level of each gene is indicated under the arrow representing that gene.

5. Summary

In summary, the present results are consistent with the idea that in protanomals, who are missing L pigment genes, the M pigment gene farthest upstream is the one expressed in the highest number of cones. In normals, an L pigment gene is first in the array [12,3] and expressed in the highest number of cones [15,16]. This may suggest that individual cones have no identity that is independent of which pigment gene is expressed within them and that the L/M identities of the cones and their distribution is dictated by the structure of the X-chromosome pigment gene array.

Acknowledgements

This work was supported by National Eye Institute grants EY09620, EY09303, and EY01931, and by Research to Prevent Blindness via an unrestricted grant to Ophthalmology at MCW, and a James S. Adams Scholar Award to M.N.

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