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Molecular genetic detection of female carriers of protan defects

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Abstract

Females heterozygous for congenital colour vision defects are of interest because they are believed to have cone photoreceptor ratios and cone photopigments that differ from normal. We describe a molecular genetic method to identify protan carriers that involves characterizing the genes that occur in the most upstream position in each of the X-chromosome photopigment gene arrays. © 1998 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Females who are heterozygous carriers of congenital colour vision defects have long been studied by researchers interested in the mechanisms underlying colour vision. Carriers can have proportions of cone photoreceptors and cone pigments in their retina that differ from normal. As a result, they provide unique opportunities to study how alterations in the cone photoreceptor mosaic affect vision. Women who are heterozygous for protanopia or protanomaly are thought to have an unusually high ratio of middle wavelength-sensitive (M) to long wavelength-sensitive (L) cones. Carriers of protanomalous trichromacy are believed to have the photoreceptor basis for tetrachromatic colour vision and have been subjects of studies which have examined the consequences of four cone types for color vision [1,2]. In the past, a researcher was obliged to demonstrate that a woman was either the mother or the child of a male with a colour vision defect to confirm that she was a carrier. Here we describe a molecular genetic method to identify protan carriers.

The M and L visual pigment genes lie in a head-to-tail tandem array on the X-chromosome [3]. We have developed a long PCR- based method to isolate and characterize the first gene in the array [4]. Nucleotide differences in exons 2, 3, 4 and 5 of the genes encode spectral differences among the pigments. Substitutions in exons 2, 3 and 4 produce relatively small shifts which can be thought of as producing spectral subtypes within the L and M pigment classes. Such spectral subtypes occur as polymorphic variants in the normal population and they occur in individuals with color vision defects. In contrast, sequences in exon 5 produce a large spectral shift which divides the pigments into distinct L and M classes. Thus, we have used the identities of sequences in exon 5 to characterize the first gene as L or M.

We characterized the first gene in the X-chromosome array of 22 men with normal colour vision (to be described elsewhere) and 27 deuteranomalous men [4,5]. All of the men had an L pigment gene in the first position. In contrast, the 22 protan men who served as controls in the present project did not have L pigment genes. They, thus, were obliged to have a gene with an M pigment-encoding exon 5 in the first position in the array. This unique feature of protan gene arrays makes them easily detectable in women who are heterozygous for protan defects. Protan carriers are predicted to have one array with an M pigment gene in the first position and the other array with an L pigment gene first. Non-carrier women would have L genes first in both arrays.

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2. Methods and materials

2.1. Subjects

Genetic testing was done on members from two unrelated families. All members from family A had normal colour vision with the exception of one son who had a protan defect. From his performance on a battery of tests, including the Rayleigh match (Nagel), D-15, Ishihara's tests, AO-HRR plates and APT-5, he was diagnosed as extreme protanomalous. All subjects from family B had normal colour vision; however, the mother and two daughters had abnormally low sensitivity to long wavelengths as measured by heterochromatic flicker photometry (HFP) [6] and thus had luminosity functions characteristic of protan carriers (Schmidt's sign; [7]). The father and one daughter had normal luminosity functions. Control subjects were 22 men identified as protan in the Rayleigh match.

2.2. DNA isolation and first gene amplification

Whole blood samples were obtained from the members of each family. Genomic DNA was isolated using the Wizard genomic DNA purification kit (Promega). The Expand Long Template PCR System (Boehringer Mannheim) was used as recommended by the manufacturer to do long PCR. The first gene in the array was amplified as described in Neitz et al. [4]. PCR products were gel purified in 0.8% low melt, SeaPlaque agarose (FMC). Prior to gel purification, gel apparatuses were treated with 10% bleach for 30 min to render any residual DNA unamplifiable [8]. DNA bands were excised from the gel and used as template to amplify exon 5. Primers and conditions were those previously used by Neitz and Neitz [9], except that here the primers were 5′ end-labeled with fluorescein.

2.3. Restriction digestion assay and DNA sequencing

The spectral difference between L and M pigments is produced by two amino acid substitutions encoded by exon 5. The codon for one of these, 277, is associated with an *Rsa*I restriction site in exon 5 of L genes that is absent in exon 5 of M genes [3,9]. PCR-amplified exon 5 from the first gene in the X-chromosome arrays was digested with *Rsa*I restriction enzyme, the digestion products were electrophoretically separated in 6% polyacrylamide and the DNA bands were visualized with the FluorImager (Molecular Dynamics). As a control, the same procedure was performed on 22 protan men, except that in this experiment exon 5 was amplified from genomic DNA instead of from the first gene in the array. The analysis therefore assayed exon 5 from all the genes in the array from each of the protans. So that control subjects were also tested with the identical protocol used on the families, we also performed the first gene in the array experiment on 11 of the 22 protans.

Exon 5 was amplified from the first gene in the arrays as described by Neitz and Neitz [9] except that here, the positive and negative strand primers were tagged with the M13 forward and reverse primer sequences, respectively for dye-primer sequencing. Direct sequencing was done with AmpliTaq FS (Perkin–Elmer- ABI) and an ABI 310 genetic analyzer.

3. Results

Our PCR strategy for isolating and characterizing the first gene in the arrays from each subject is illustrated in Fig. 1. A primer that hybridized only to sequences upstream of the first gene in each array was paired with a non-selective primer that hybridized to sequences in intron 5. This approach allowed us to amplify the first gene in each array, regardless of whether it encoded an L or an M pigment. A 13 kb DNA fragment containing exons 1–5 of the first gene in each array was obtained and, following gel purification, was used as template in PCR to amplify exon 5 from the first gene (Fig. 1).

A *Rsa*I restriction digestion assay was used to characterize exon 5 from the first gene in the arrays for each subject. If a woman has an L pigment gene in the first position in the array on both of her X-chromosomes, only DNA fragments corresponding to *Rsa*I cleaved exon 5 will be observed. However, if a woman has one array with an M gene in the first position and a second array with an L gene first, then fragments corresponding to intact exon 5 and *Rsa*I cleaved exon 5 will be observed.

The results of the *Rsa*I restriction digestion assay are shown in Fig. 2a. Each lane in the gel is aligned with the person in the pedigree whose DNA was contained in that lane. The colour normal son and daughter in family A showed the identical banding pattern. For both, only DNA fragments corresponding to L pigment genes were observed. For the protan son (labeled 'P' in the pedigree), only the DNA fragment corresponding to an M pigment gene was observed. A total of 22 unrelated protan men had the same result when, instead of the first gene, all the genes in the array were analyzed—none had an array that showed evidence for a gene with an L pigment-encoding exon 5. From this we concluded that they all had an M pigment gene first in the array. Nonetheless, to be consistent in our methodology we performed the first gene in the array analysis on 11 of the 22 protans. They all had only the fragment corresponding to an M pigment gene, identical to the result from the protan son in family A.

The obligate carrier in family A ('OC') was heterozygous. The banding pattern observed showed both

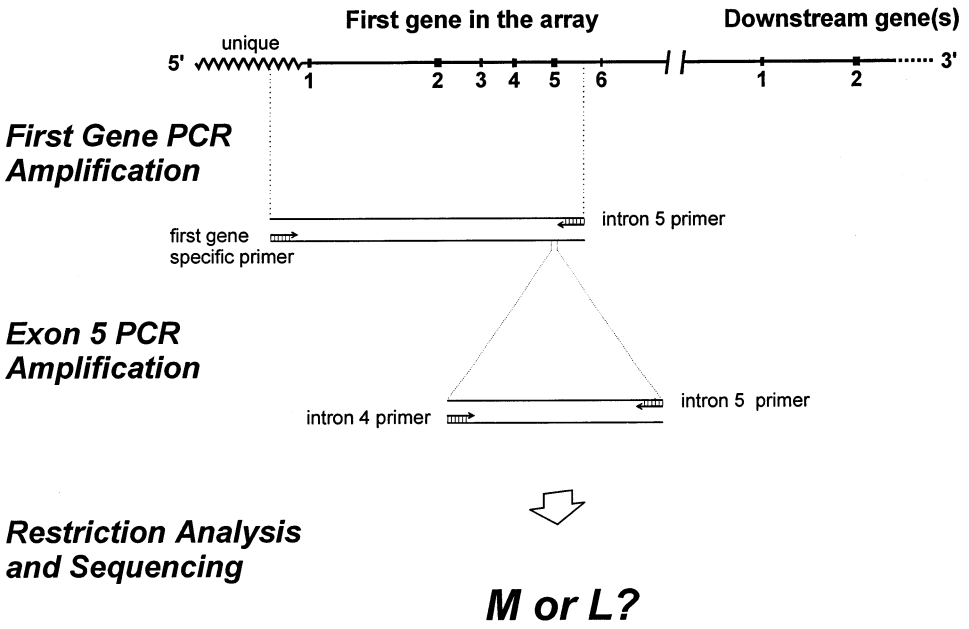

Fig. 1. Strategy to amplify and characterize the first gene in the X-chromosome visual pigment gene array. A hypothetical pigment gene array is shown with the exons of each gene numbered. The primers and their specificities are described in the methods. The first gene PCR product was gel purified and used as template to amplify exon 5. Restriction digestion analysis and DNA sequencing were used to determine whether exon 5 from the first gene encoded an M or an L pigment.

L and M pigment gene fragments. In family B, the mother and the two daughters with protan luminosity functions ('PL') were suspected to be protan carriers. The banding patterns observed for all three showed that each woman had both L and M gene fragments. The daughter with the normal luminosity function had a banding pattern that corresponded to the presence of only L pigment gene fragments.

The DNA sequence of exon 5 from the first gene in the arrays from each subject was determined to confirm the restriction digestion assay results. Examples of the sequencing results are shown in Fig. 2b. Each peak in the electropherogram corresponds to a single nucleotide. The four possible nucleotides are each represented by a different line pattern. In the top electropherogram, exon 5 from the first gene of the colour normal son from family A reads GTACT, corresponding to nucleotide positions 1322–1326 (numbering system from Nathans et al. [3]). This encompasses one of the two major sites responsible for the difference between L and M pigments. The colour normal and protan sons differed in exon 5 of the first gene at positions 1322 and 1324. These were G and A respectively for the normal son (top panel, Fig. 2b), but A and T, for the protan son (middle panel, Fig. 2b). In addition to the sequence shown in Fig. 2b, exon 5 from the two sons showed all the typical differences between L and M.

In a woman, two peaks of similar height at one location in the electropherogram indicated that exon 5 from the first gene in each array differed at that position in exon 5. The bottom panel in Fig. 2b shows the result from one carrier as an example: two peaks were evident at position 1322 and position 1324 (G/A and A/T, respectively). All females with both M and L exon 5 fragments in the restriction digestion assay showed two peaks at both the locations that distinguish M from L genes. All other subjects had the same DNA sequence as the colour normal male in Fig. 2b.

4. Discussion

The women in the present study who were obligate carriers of a protan defect or who had a protan luminosity function were shown to have one X-chromosome array with an L pigment gene in the first position and one array with an M pigment gene first. The only daughter in family B with a normal luminosity function had L genes in the first position in both of her X-chromosome arrays. She and the daughter in family A had a 50% chance of inheriting the defective gene array from their mothers, but the molecular genetic data suggests that they did not. Overall the results suggest that this test can reliably identify female carriers of protanopia or protanomaly without relying on the presence of an affected family member.

A limitation of our method is the possibility that not all protan gene arrays have an M pigment gene first. One example has been reported where L gene sequences were found in the array of a man with a protan defect [10]. If an L gene were first in this protan array, the

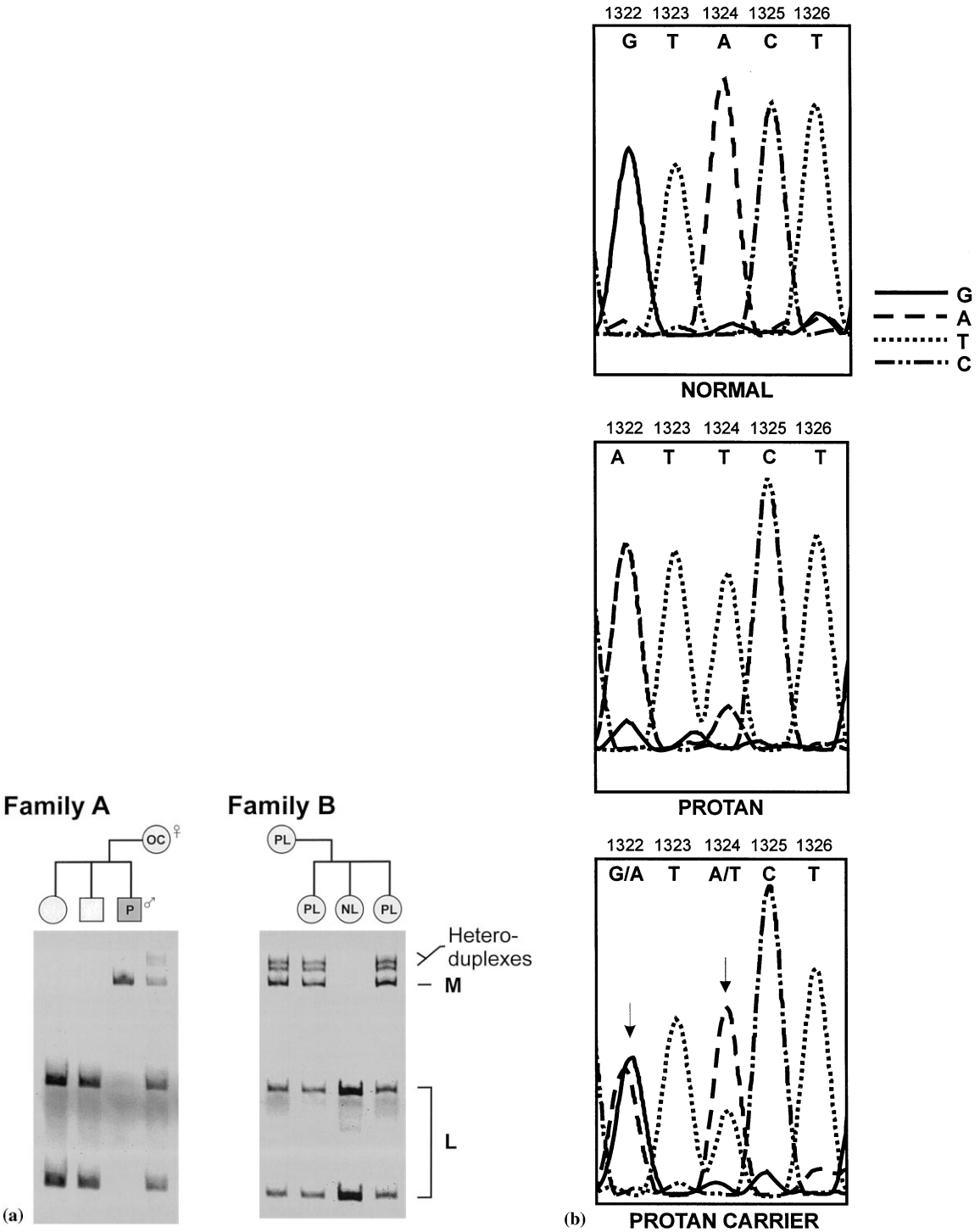

Fig. 2. (a) Pedigrees and molecular genetic results from two families. In the pedigrees, the circles represent females and the squares represent males. In family A, OC is an obligate carrier, P is a protan colour defective male. In family B, NL had a normal luminosity function and PL individuals had a protan luminosity function (see methods). Shown below the pedigrees are fluorimages of the *Rsa*I restriction digestion assay. Each lane in the gel is aligned with the family member in the pedigree whose DNA is contained in that lane. L indicates two bands that are the products of *Rsa*I cleavage of exon 5 from L genes, M indicates the band derived from M genes which is not cleaved by *Rsa*I. Heteroduplexes are formed when both M and L gene fragments are present [9]. (b) DNA sequence electropherograms showing nucleotide positions 1322 to 1326 in exon 5 from the first gene for a colour normal male (top), a protan male (middle) and a female carrier (bottom). Position 1322 and 1324 are the two nucleotides that distinguish an M from an L exon 5 sequence. Arrows indicate nucleotide positions where two peaks are present in sequence obtained from the female carrier.

female carrier of such an array would go undetected by our method. Such cases are expected to be rare. In theory, there may also be a gene array that confers normal colour vision, has both M and L genes, but has an M gene first. This has not been observed, but if it did occur, women who carried such arrays would be incorrectly diagnosed as protan carriers. Having a valid method that can quickly identify females heterozygous for colour vision defects can be used to identify subjects for studies of the mechanisms underlying colour vision.

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