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ORIGINAL INVESTIGATION

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A new mechanism in blue cone monochromatism

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Abstract Blue cone monochromatism (BCM) is a rare X-linked colour vision disorder characterized by the absence of both red and green cone sensitivity. Most mutations leading to BCM fall into two classes of alterations in the red and green pigment gene array at Xq28. In one class the red and green pigment genes are inactivated by deletion in the locus control region. In the second class genetic rearrangements have created an isolated pigment gene that carries an inactivating point mutation. Here we describe a clinical case of BCM caused by a new mutation where exon 4 of an isolated red pigment gene has been deleted. The finding represents the first intragenic deletion yet described among red and green pigment genes.

Introduction

Normal colour vision is trichromatic and is mediated by the blue, green and red visual pigments present in the corresponding populations of blue, green and red photoreceptor cells (cones) of the retina. These three classes of photoreceptors have maximum sensitivity for light at 430 nm (blue), 535 nm (green) and 565 nm (red). The blue visual pigment is coded for by a gene located on chromosome 7 (Nathans et al. 1986b). The red and green visual pigments are coded for by the highly homologous red and green pigment genes located distally on the long arm of the X chromosome (q28) in a head-to-tail tandem array (Nathans et al. 1986a). This arrangement has predisposed this visual pigment gene locus to frequent illegitimate recombination or gene conversion between the red and green pigment genes. These types of events are responsible for almost all inherited variations in red-green colour discrimination and include deletion of the green pigment genes and generation of red-green and green-red hybrid genes (Nathans et al. 1986b; Deeb et al. 1992; Winderickx et al. 1993).

Blue cone monochromatism (BCM), or X-chromosome-linked incomplete achromatopsia, is a rare condition characterized by absence of red and green cone function (Pokorny et al. 1979). Daylight colour vision is mediated only by the blue cones, and without a comparison between different classes of photoreceptors blue cone monochromats cannot discriminate between light of different wavelengths and light intensity. Therefore, these individuals have poor or no colour discrimination and perceive the world colourless. Mutations in the red and green pigment gene array that destroy production of functional red and green pigments and thus inactivate the corresponding cones have been identified in the great majority of BCM cases studied (Nathans et al. 1989, 1993).

The characteristic clinical feature of BCM, besides poor colour discrimination, is reduced central vision with concomitant nystagmus. Congenital nystagmus is a presenting symptom in several hereditary congenital retinal dysfunctions including Leber's congenital amaurosis (Heher at al. 1992), congenital stationary night blindness (Weleber and Tongue 1987), Åland eye disease (Forsius and Eriksson 1964), achromatopsia (Francois et al. 1959), blue cone monochromatism (Blackwell and Blackwell 1961), oligo-cone-trichromacy (Van Lith 1973), and cone dysfunction with supernormal rod response (Rosenberg and Simonsen 1993). Some of these conditions may be associated with colour vision deficiencies.

The recently constructed blue cone monochromatism plates (Berson et al. 1983) have become an important aid to differentiate between BCM and complete achromatopsia, and by introduction of electroretinographical blue cone recordings the clinical diagnosis of BCM has been improved (Gouras and MacKay 1990). At the molecular level mutation analyses introduced by Nathans and collaborators (Nathans et al. 1993) have proved highly efficient as key answers to the clinical diagnosis of BCM.

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The mutations in the red and green pigment gene array causing BCM are found to be of two classes. In the first class a normal red and green pigment gene array has been inactivated by a deletion in the locus control region located 5′ of the red pigment gene. A deletion in this region abolishes transcription of the genes in the locus and therefore inactivates both red and green cones (Wang et al. 1992). In the second class of mutations the locus control region is preserved but rearrangements within the red and green pigment gene array inactivate the locus. The most common genotype in this class consists of an isolated inactive red-green hybrid gene created by intragenic unequal crossing over between a normal red pigment gene and an inactive green pigment gene normally present in the gene array of 2% of Caucasian males. This green pigment gene carries an inactivating thymine-to-cytosine transition at nucleotide 648, which results in a cysteine-to-arginine substitution at codon 203 (Winderickx et al. 1992; Nathans et al. 1993).

We examined Danish BCM patients and found a new type of BCM mutation. We report here on the clinical and molecular findings.

Materials and methods

Ophthalmological examinations were performed at The National Eye Clinic for the Visually Impaired as outlined in the Result section.

High molecular genomic DNA was isolated by standard methods from peripheral blood of the proband with BCM, a brother with normal colour vision, a maternal halfbrother with BCM, the carrier mother (Fig. 3) and from peripheral blood of an unrelated colour vision normal male and an unrelated deuteranope male having an isolated normal red pigment gene. Southern blot analysis of restriction fragments of genomic DNA, digested with the restriction enzymes *Rsa*I and a combination of *Eco*RI and *Bam*HI, was done as previously described (Drummond-Borg et al. 1989; Jørgensen et al. 1990). The E and F restriction fragments (Fig. 1) were visualized on Southern blots using a cDNA probe (hs7 large fragment) containing sequences from the 3′ half of exon 2 and the remaining exons 3 through 6 (Nathans et al. 1986b).

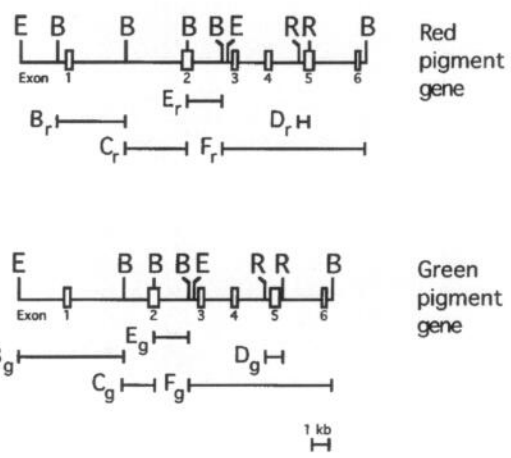

Fig. 1 Restriction maps of red and green pigment genes showing the characteristic fragments visualized by Southern blotting. *E*, *B* and *R* indicate the recognition sites of restriction endonucleases *Eco*RI, *Bam*HI and *Rsa*I, respectively. Fragments originating from the red pigment gene are designated with subscript *r* and the corresponding fragments from the green pigment gene with subscript *g*

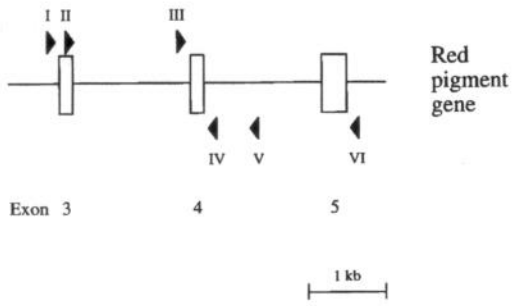

Fig. 2 Part of the red pigment gene. *Arrowheads* indicate orientation (5′→3′) and locations of primers used for PCR amplification and sequence analysis. For primer description see Materials and methods

PCR amplification and cloning

The localization of the PCR primers used are shown in Fig. 2. All PCR primers are described by Nathans et al. (1993).

The ~3.7-kb normal fragment extending from exon 3 through exon 5 of the red and green pigment genes of the normal X chromosome and the ~2.1-kb deletion fragment of the BCM-causing X chromosome were amplified using primer I (JN-289, Nathans et al. 1993) and primer VI (JN-455, Nathans et al. 1993). In addition, two overlapping PCR fragments from a male with an isolated normal red pigment gene were amplified. One fragment containing exon 3, intron 3 and exon 4 was amplified using primer I and primer IV (JN-280, Nathans et al. 1993). The second fragment containing exon 4, intron 4 and exon 5 was amplified using primer III (JN-279, Nathans et al. 1993) and primer VI. PCR was done using the Stratagene *Taq* extender system in a reaction volume of 50 µl containing 200 ng genomic DNA and 50 pmol of each primer and a concentration of 200 µM of each dNTP. Amplification conditions for each of 30 cycles were: 94°C for 1 min, 59/60°C for 1 min and 72°C for 2.5 min. The PCR fragments were cloned as either *Hin*dIII/*Hin*dIII or *Hin*dIII/*Eco*RI fragments in the M13mp18 vector and sequenced.

Sequencing

Cycle sequencing was performed on an automatic DNA sequencer (ABI373A) using fluorophore-labeled dideoxy terminators as recommended by the manufacturer (Perkin Elmer). Single-stranded mp18 recombinant clones were sequenced using M13 sequencing primer, a primer (primer II, Fig. 2) located at base pairs 112–127 in the red pigment gene near the exon 3-intron 3 junction (Nathans et al. 1986a) and a primer (primer V, Fig. 2) located at base pairs 455–471 in intron 4 of the red pigment gene (Shyue et al. 1994). With these primers it was possible to sequence both DNA strands across the fusion point in the deletion fragment and to obtain intron 3 sequences from a normal red pigment gene.

Results

History and clinical findings

The proband was born in 1978. He was delivered by Caesarian section 4 weeks preterm due to low maternal estri-

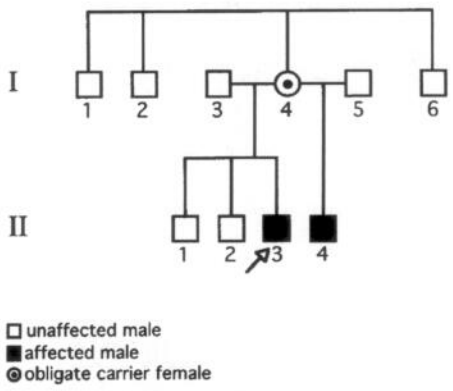

Fig. 3 Pedigree of a family in which X-linked blue cone monochromatism segregates. *Arrow* indicates the proband

ole values. The neonatal period was complicated by respiratory distress. The parents and two elder brothers (Fig. 3) were healthy and had no visual complaints. There was no history of colour blindness in the maternal family. A maternal uncle was mentally retarded.

Nystagmus was observed at the age of 5 months and interpreted to be the result of minor brain dysfunction. The first ophthalmological examination was done in 1983 and revealed a horizontal pendular nystagmus, hypermetropia and reduced visual acuity (VA). Neurological examination at the age of 5 years was unremarkable and a CT brain scan demonstrated only a slightly asymmetric ventricular system.

At the first examination at the National Eye Clinic for the Visually Impaired in 1985, the VA was 6/36 on both eyes. A moderate hypermetropia and astigmatism was found. Ocular albinism was suspected due to partially pellucid irides. Yet, colour vision tests disclosed incomplete achromatopsia. Scotopic Ganzfeld electroretinography (ERG) demonstrated a normal single flash rod response although the oscillatory potentials were reduced. Totally absent 32 Hz flicker responses was in accordance with a diagnosis of congenital achromatopsia or a cone dystrophy. Visual evoked cortical potentials had reduced amplitudes and normal implicit times. Ophthalmoscopy revealed indistinct foveal reflexes, a sparsely pigmented fundus and a left hypoplastic optic nerve head.

At successive examinations in 1988, 1989 and 1994 a gradual shift in refractive state towards myopia was observed. No photoaversion was reported. He did well with Berson's blue cone colour plates (Berson et al. 1983), and, finally, blue cone ERG recordings with a slightly modified Gouras technique (Gouras and MacKay 1990) demonstrated preserved blue cone responses (Fig. 4).

In 1985 the mother in a second marriage gave birth to another son who had the same ophthalmological findings as his elder halfbrother. Thus both the clinical findings and the pattern of inheritance are compatible with the presence of X-linked BCM. The mother was blond with blue eyes and partially tranlucent irides. Her ophthalmological examination showed normal VA and normal colour vision (Nagel anomaloscope).

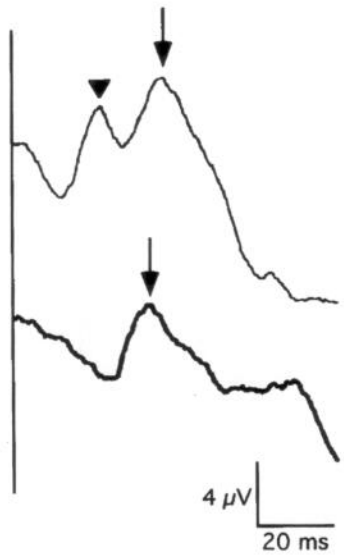

Fig. 4 Ganzfeld electroretinograms from a normal trichromat (*upper recording*) and from the patient with blue cone monochromatism (*lower recording*). The recordings were performed under photopic conditions with a yellow background and a blue flash stimulus. Each curve represents about 500 averaged stimuli. The *arrows* indicate the blue cone responses, which are present in both persons, while the red-green cone response (*arrowhead*) is present in the colour vision normal person only

Molecular findings

The restriction fragments used to study the red-green colour vision locus are shown in Fig. 1. The banding pattern of DNA from an unrelated male with normal colour vision is seen in the left lane of the Southern blot in Fig. 5A. The pattern and the equal density of corresponding bands from red and green pigment genes indicate that the locus contains one normal red pigment gene followed by one normal green pigment gene. A dramatically changed banding pattern is seen in the right lane of Fig. 5A, where DNA from the proband (II-3, Fig. 3) is analysed. All fragments originating from the green pigment gene(s) are missing, which means that only an isolated red pigment gene remains at the locus. With no green visual pigment produced the predicted phenotype is deuteranopia. However, the clinical diagnosis is BCM indicating that neither green nor red functional visual pigments are produced. We reasoned that the remaining red pigment gene carried an inactivating mutation.

An inactivating amino acid substitution, C203R (cysteine residue at codon 203 substituted by an arginine), has recently been identified in a BCM patient carrying an isolated and otherwise normal red pigment gene (Nathans et al. 1993). In the search for this mutation we tried to amplify exon 4 by PCR, but the amplification failed. Subsequent PCR analysis confirmed the presence of both exon 3 and exon 5 (data not shown). However, no PCR product was obtained with combinations of exon 3 and exon 4 primers (primers I and IV, Fig. 2) and of exon 4 and exon

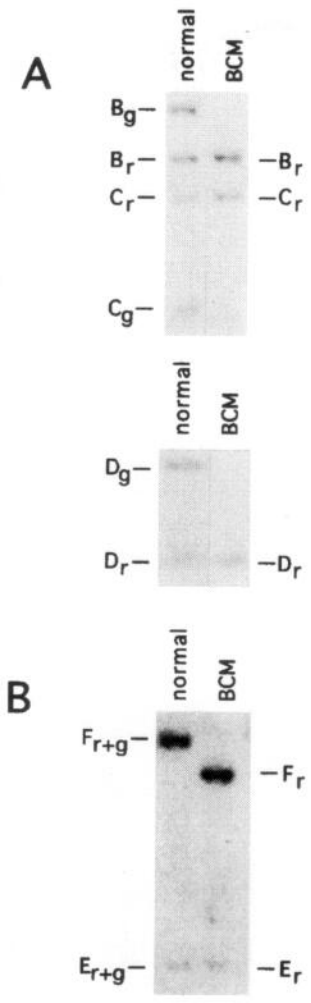

Fig. 5A–B Southern blots of genomic DNA from an unrelated male with normal colour vision (*left lane*) and from the proband (II-3) with blue cone monochromatism (BCM; *right lane*). The bands B_g, B_r, C_r, C_g, and D_g and D_r in (**A**) and E_{g+r} and F_{g+r} in (**B**) correspond to the restriction fragments shown in Fig. 1. Note the absence of bands representing the green pigment gene in the BCM lane. The F_r-fragment in the BCM lane is ~1.6-kb shorter than the normal F_r-fragment

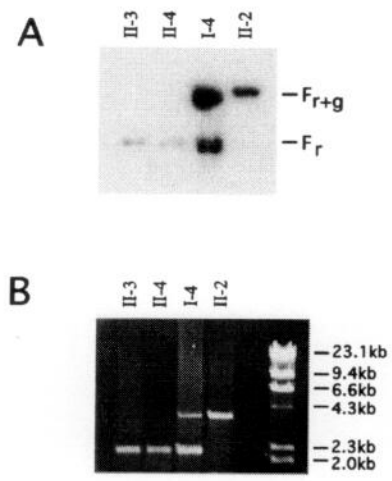

Fig. 6 **A** Southern blot of DNA from proband II-3 with BCM (*lane II-3*), his halfbrother II-4 with the same BCM phenotype (*lane II-4*), his colour vision normal brother II-2 (*lane II-2*) and the colour vision normal carrier mother I-4 (*lane I-4*). II-3 and II-4 share the same shortened F-fragment, which they have received from their heterozygote mother, whereas II-2 has received the X chromosome with the full-length F-fragment. For description of restriction fragment and probe used see Fig. 1 and Materials and methods. **B** Agarose gel electrophoresis of PCR products of DNA from individuals *II-3*, *II-4*, *II-2* and *I-4* as indicated above the lanes. The primers used are I and VI as indicated in Fig. 2. The ~3.7-kb PCR fragment in II-2 represents the normal internal fragment of the red and green pigment genes. This PCR fragment is only ~2.1-kb in II-3 as a result of a ~1.6-kb internal deletion of the red pigment gene. The same deletion fragment is present in II-4. Both the normal ~3.7-kb fragment and the ~2.1-kb deletion fragment are present in I-4. For details, see text

5 primers (primers III and VI). Furthermore, Southern blot analysis revealed a ~1.6-kb deletion in the F-fragment of the red pigment gene (Fig. 5B, right lane). These results indicate that the ~1.6-kb sequences deleted begin in intron 3 and end in intron 4 leaving intron 3 sequences in direct continuation with intron 4 sequences. The same shortened F-fragment and no full-length F-fragment were found in DNA of the maternal halfbrother with BCM (II-4, Fig. 6A), indicating that his (II-4) locus contains no normal red or green pigment genes but has the same abnormal structure as the locus of the proband (II-3). By contrast, the full-length F-fragment and no shortened F-fragment was observed in DNA of the colour vision normal brother (II-2, Fig. 6A). Both the shortened F-fragment of the X chromosome carrying the BCM mutation and the full-length F-fragment of the X chromosome carrying a normal locus were observed in the colour vision normal carrier mother (I-4, Fig. 6A). These results were confirmed by PCR amplification of a fragment extending from exon 3 through exon 5 of the red and green pigment genes, defined by primers I and VI as indicated in Fig. 2.

The results, shown in Fig. 6B, demonstrate that the carrier mother (I-4) shares the normal ~3.7-kb fragment with her colour vision normal son (II-2), while she shares the deletion fragment (~2.1-kb) with her two sons (II-3, II-4) who have the same BCM phenotype.

To identify the exact fusion point we cloned a fragment extending from exon 3 through exon 5. For comparison we cloned the corresponding normal sequences: one fragment containing exon 3, intron 3 and exon 4 sequences and an overlapping fragment containing exon 4, intron 4 and exon 5 sequences. These fragments originated from an unrelated deuteranope male having an isolated normal red pigment gene. To select a sequencing primer, we designed a series of intron 4 primers according to a published sequence (Shyue et al. 1994) and chose a primer (primer V, see Fig. 2) located 455 bp downstream of exon 4 and close to the deletion breakpoint. This primer V and the exon 3 primer II (see Fig. 2) were used to sequence both strands across the fusion point.

Figure 7 shows the result of the sequence analysis. The first 353 nucleotides of the intron 3 sequences of the BCM deletion gene are identical to the normal sequence. At position 354 this identity ends abruptly and the sequence continues as intron 4 sequences. The nucleotide at position 354 (C, see Fig. 7) and the following 265 nucleotides sequenced are identical to normal intron 4 sequences beginning 189 nucleotides downstream of exon 4. The three preceding nucleotides at positions 351–353 in intron 3

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