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Genetics

Selective expression of human X chromosome-linked green opsin genes

(visual pigments/opsin mRNA expression/selective gene expression)

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ABSTRACT **The human red and green photopigments are specified by genes on the long arm of the X chromosome (Xq28). In individuals with normal color vision, the locus was proposed to consist of a single red pigment gene upstream of one or more copies of green pigment genes. The presence of a single red pigment gene in the array was confirmed by demonstration of only one retinal mRNA transcript coding for the red opsin. In individuals with multiple green pigment genes, it is unknown whether all genes are expressed. We analyzed the sequence of red- and green-specific mRNA from retinas of individuals with multiple green pigment genes in comparison with the corresponding genomic DNA sequences. The data showed that only a single green pigment gene is expressed. We therefore suggest that a locus control-like element, already known to be located 3.8 kilobases upstream of the transcription initiation site of the red pigment gene, allows transcription of only a single copy of the green pigment genes, probably the most proximal copy. This finding provides an explanation for the not-infrequent presence of 5′ green–red hybrid genes in individuals with normal color vision. Such hybrid genes are usually associated with defective color vision. We suggest that 5′ green–red hybrid genes produce defective color vision only when their position in the gene array allows expression in the retinal cone cells.**

Normal human color vision is mediated by the red (long-wavelength sensitive) and green (middle-wavelength sensitive) visual pigments present in the corresponding red- and green-sensitive cone cells of the retina. The genes that encode the apoproteins (opsins) of these pigments are located on the long arm of the X chromosome (q28) and are arranged in a head-to-tail tandem array with a single red opsin gene upstream of one or more copies of green opsin genes (1–3). Defects and anomalies in red/green color vision occur in ≈8% of the Caucasian male population. They are almost always associated with deletion of the green opsin genes or presence of 5′ red–green or 5′ green–red hybrid genes, most likely the result of unequal recombination between the highly homologous genes at this locus (4, 5). Rarely, red/green color vision defects are associated with substitution of a conserved cysteine by an arginine residue at position 203 (C203R) in the green opsin (6, 7). The 5′ red–green hybrid genes may replace the normal red opsin gene and are always expressed as a protan type of color vision deficiency. The 5′ green–red hybrid genes, however, do not always lead to color vision deficiency. We observed that the frequency of 5′ green–red hybrid genes is higher than the frequency of phenotypic color vision defects among Caucasians and in particular among Afro-Americans (8, 9). Recently, we demonstrated unequivocally that 4 of 129 Caucasian males with anomaloscopically proven normal color vision had a 5′ green–red hybrid gene in addition to normal red and green opsin genes (5). We also found an individual with normal color vision who had the critical C203R mutation in only one of his five green opsin genes (7). Thus, 5′ green–red hybrid genes and mutant green opsin genes are associated with normal as well as abnormal color vision. To explain these observations, we suggested that not all the genes in a red/green opsin gene array are expressed. We hypothesized that green-sensitive cones express either a 5′ green–red hybrid or mutant green opsin gene or a normal green opsin gene but not both.

To test this hypothesis, we made use of a common silent polymorphism (A vs. C at the third position of codon 283) in exon 5 of the green opsin gene. Approximately 74% of males with more than one green opsin gene were found to have both alleles (J.W., Y. Hibiya, A.G.M., and S.S.D., unpublished data). Furthermore, the two alleles, henceforth referred to as A and C, could easily be distinguished by single-strand conformation polymorphism (SSCP) analysis (10, 11) or by digestion with *Eco*O109I.

METHODS

Preparation of DNA and RNA. Retinal tissue and blood samples were collected between 6 and 8 hr after death. Specimens from 6 Caucasian male subjects (CV2507–CV2792) were obtained through the Lions Eye Bank of the University of Washington and from 7 subjects (CV2819–CV2825) through the University of Barcelona. Genomic DNA was prepared from peripheral blood leukocytes as described (12). Total RNA was extracted from retinas (stored at −70°C) by the method of Chomczynski and Sacchi (13). cDNA was prepared from retinal mRNA using SuperScript RNase H⁻ reverse transcriptase (GIBCO/BRL). Two samples were prepared for each subject, using as primer either a random hexanucleotide or an oligonucleotide complementary to the 3′ border of exon 5 of the red and green opsin genes (primer 10 in Table 1).

Southern and Northern Blot Analysis. Southern blot analysis was used to determine the gross structure of the red–green pigment gene array as described (4, 5). Northern blot analysis (14) was used to characterize mRNA transcripts of the red and green pigment genes in retinal tissues. The probe used to detect these transcripts was a 350-base-pair (bp) cDNA fragment encompassing exon 1 and part of exon 2 of the red pigment gene kindly provided by J. Nathans (4). The Northern blot was also hybridized with a 700-bp fragment of the chicken β-actin cDNA (15).

Sequence Analysis of Genomic DNA and cDNA. Sequence analysis of exon 5 of the red and green opsin genes was performed by PCR amplification followed by SSCP analysis or restriction enzyme digestion. The sequence of all oligonucleotide primers is given in Table 1. A segment encompassing part of exons 4 and 5 of the red or green opsin cDNA

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Abbreviations: SSCP, single-strand conformation polymorphism; LCR, locus control-like region.

Table 1. Sequence of oligonucleotide primers

Number	Sequence (5′ → 3′)	Location
1	GCTGCATCATCCCACTCGC	Exon 4 (R)
2	GCTGCATCACCCCACTCAG	Exon 4 (G)
3	CTGCCGGTTCATAAAGACATAG	Exon 5
4	GTGGCAAAGCAGCAGAAAGAG	Exon 5
5	TGATGGTCCTGGCATTCTGCT	Exon 5 (G)
6	GACGCAGTACGCAAAGATC	Exon 5 (R)
7	GAAGCAGAATGCCAGGACC	Exon 5 (G)
8	CACATCGCTCCCAGATGGGT	Exon 2
9	ACACAGGGAGACGGTGTAGC	Exon 2
10	CTGCCGGTTCATAAAGACATAG	Exon 5

Locations of most primers are also indicated in Figs. 2 and 3. Primers labeled with R or G are specific for red or green opsin gene sequences, respectively. The other primers are common to both red and green gene sequences.

was amplified in a first PCR of 30 cycles of 1 min at 94°C and 1 min at 64°C, using gene-specific primer 1 or 2 with the common primer 3. The amplified product was then used as a template in a second PCR amplification (30 cycles of 1 min at 94°C and 1 min at 64°C) to amplify exon 5 by using primers 3 and 4. Exon 5 was also amplified by using genomic DNA as a template. A second strategy was used to directly amplify exon 5 (35 cycles of 1 min at 94°C and 1 min at 66°C; hot start) by using primers 5 (green specific) and 3 (common to red and green). The sequence of the amplified fragment was then analyzed by SSCP and restriction enzyme digestion.

Analysis of exon 5 sequences by SSCP was performed essentially as described (10, 11). The single-stranded DNA sequences were separated on a 5% nondenaturing polyacrylamide gel containing 10% (vol/vol) glycerol. Electrophoresis was carried out at 45 W for 6 hr at 47°C. Restriction enzyme analysis of exon 5 sequences was done by digestion with both *Eco*O109I and *Rsa* I. *Rsa* I cuts only red-specific exon 5, whereas *Eco*O109I cuts both the red and the green C allele-specific exon 5 sequences. The fragments were separated on a 6% polyacrylamide gel.

A DNA segment encompassing exons 2, 3, 4, and the 5′ portion of exon 5 of the red or green opsin cDNA was amplified (35 cycles of 1 min at 94°C, 1 min at 64°C, and 2 min at 72°C) by using primers 8 (common primer in exon 2) and either 6 (red specific in exon 5), or 7 (green specific in exon 5). The amplified fragment was then used as a template to amplify exon 2 (35 cycles of 1 min at 94°C and 1 min at 64°C) by using primers 8 and 9 (common to both red and green). The amplified exon 2 was then subjected to SSCP analysis as described above.

RESULTS

Selective Expression of Green Opsin Genes. We previously detected a silent sequence polymorphism (C vs. A) in exon 5 of the green opsin gene (J.W., Y. Hibiya, A.G.M., and S.S.D., unpublished data). Approximately 74% of Caucasian males who have more than one green opsin gene have both the A and the C alleles. We made use of this polymorphism to test whether all green opsin genes in an array are expressed into mRNA in the retina. We therefore analyzed the sequence of green opsin mRNA species in retinas of individuals who have both the A and C alleles in their genomic DNA. We collected postmortem retinal tissue (for RNA analysis) and blood specimens (for DNA analysis) from 13 unselected Caucasian males. These subjects had grossly normal red/green opsin gene arrays (no gene deletions or hybrid genes) as ascertained by Southern blot analysis. Two of them had one and 11 had two or more green opsin genes in addition to a single red opsin gene. Both the A and C green opsin alleles were present in genomic DNA of 10 of the 11 individuals with more than one green opsin gene (Table 2).

Table 2. Number and expression of green opsin genes

Subject	Number of green opsin genes	Green opsin alleles present			
		Genomic DNA		mRNA	
		G(A)	G(C)	G(A)	G(C)
CV2507	1	+	−	+	−
CV2534	2	+	−	+	−
CV2759	3	+	+	+	−
CV2760	3	+	+	+	−
CV2791	3	+	+	+	−
CV2792	2	+	+	+	−
CV2819	1	−	+	−	+
CV2820	2	+	+	+	−
CV2821	2*	+	+	+	−
CV2822	2	+	+	+	−
CV2824	2*	+	+	+	−
CV2825	3	+	+	+	−
CV2826	2*	+	+	+	−

Number of green opsin genes in the X chromosome-linked gene array was estimated by densitometric analysis of autoradiographs of Southern blots as described (4, 5). Numbers represent average ratio between intensities of restriction fragments derived from green opsin genes and those from red opsin gene. The presence (+) or absence (−) of different green opsin exon 5 sequences, A and C, in genomic DNA or mRNA was determined by PCR amplification followed by SSCP analysis or *Eco*O109I/*Rsa* I digestion as described.
*Numbers may have been underestimated because of DNA degradation.

Northern blot analysis of total retinal RNA revealed an abundant message ≈1.45 kilobases (kb) long (Fig. 1) that represented a mixture of red and green opsin mRNAs. cDNA was prepared from retinal RNA and used as a template to amplify exon 5 of either the red or the green opsin gene with gene-specific primers. Exon 5 was also amplified, with genomic DNA used as a template. The sequence of the amplified fragments was analyzed by SSCP and by *Eco*O109I/*Rsa* I digestion (Fig. 2). The results showed clearly that whenever the two variants of exon 5 of the green opsin gene, A and C, were detected in genomic DNA, only one variant was detected in retinal RNA (Table 2). It is remarkable that A was always the expressed allele. This observation could not be due to reverse transcription or PCR amplification artifacts, since we obtained similar results by using two different primers to generate cDNA and two different strategies for gene-specific amplification of exon 5 (Fig. 2).

Expression of the Red Opsin Gene. Retinal RNA (isolated from the 13 males described above) was analyzed for red opsin encoding sequences in order to test the hypothesis that more than one red opsin gene is expressed in the retinas of

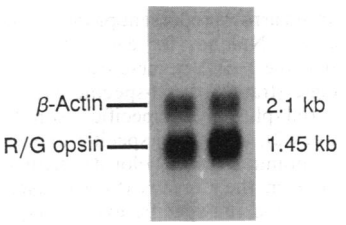

FIG. 1. Autoradiograph of a Northern blot hybridized with human red pigment cDNA and chicken β-actin cDNA. Densitometric analysis of the autoradiograph showed that the signal for red/green (R/G) opsin mRNA was 3–4 times stronger than that of β-actin. Taking into account that the β-actin probe is twice as long as the pigment probe and assuming that both probes hybridized equally well, this would indicate that the red/green opsin mRNA is 6–8 times as abundant as the β-actin mRNA in the retina.

most individuals with normal color vision (16, 17). cDNA prepared from total retinal RNA was used as a template to amplify by PCR red opsin-specific fragments encompassing exons 2, 3, 4, and the 5′ portion of exon 5 by using primers 6 (red specific in exon 5) and 8 (common to both red and green in exon 2). The amplified fragment was then used as a template to amplify exon 2 by using primers 8 and 9 (see Fig. 3*A* for amplification strategy). The amplified exon 2 fragments were then analyzed for sequence variation by SSCP.

The results showed that only a single red opsin mRNA species was present in retinal RNA of all 13 subjects (Fig. 3*B*). The deduced amino acid residues at codons 65, 111, and 116 in exon 2 of this mRNA correspond to those characteristic of the red opsin gene. Since exon 5 of this mRNA transcript was also red opsin specific, the expression of any additional red pigment genes [including green–red hybrid genes (16, 17)] in any of the 13 subjects is excluded. These results are in complete agreement with those described in Fig. 2, in which a single sequence corresponding to exons 4 and 5 of the red opsin mRNA was detected in retinal tissues of all subjects.

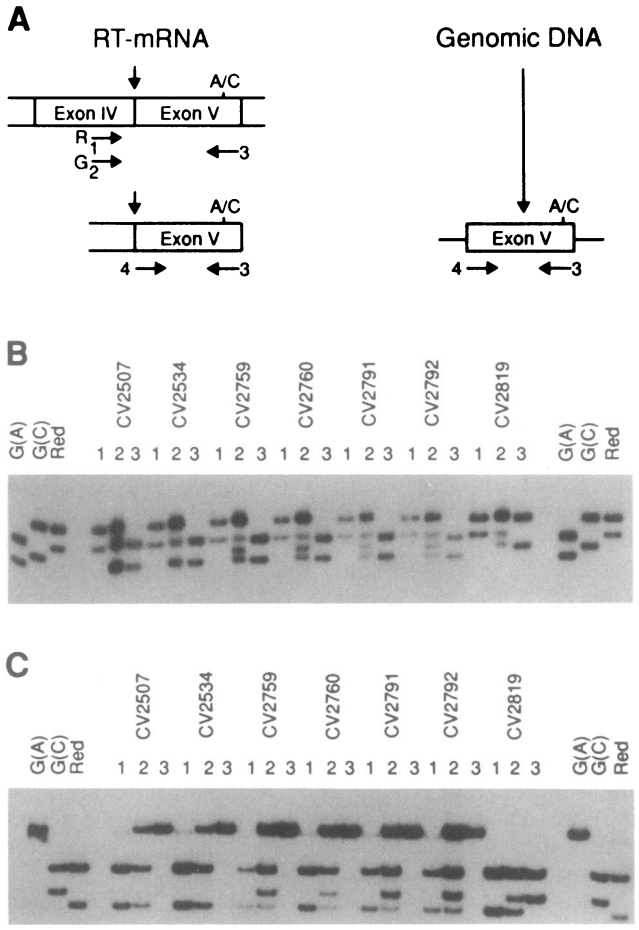

FIG. 2. Sequence analysis of genomic and cDNA red and green exon 5 fragments. (*A*) PCR amplification strategy. Arrows R or G represent, respectively, primers specific for red or green opsin sequences. Unmarked arrows represent primers common to both red and green sequences. Number for each arrow refers to primer sequence given in Table 1. RT, reverse transcribed. (*B*) Autoradiograph showing single strands of red-specific exon 5 amplified from cDNA (lanes 1), red plus green-specific exon 5 amplified from genomic DNA (lanes 2), and green-specific exon 5 amplified from cDNA (lanes 3). Genomic DNA of color-deficient subjects who had only one opsin gene in their arrays (5) was used to amplify the controls G(A), G(C), and red-specific exon 5 sequences shown on both ends of the gel. SSCP was performed essentially as described (10, 11). Single-stranded DNA sequences were separated on a 5% nondenaturing polyacrylamide gel containing 10% glycerol. Electrophoresis was carried out at 45 W for 6 hr at 47°C. (*C*) Restriction enzyme analysis of exon 5 sequences. Autoradiograph shows PCR-labeled exon 5 sequences digested with both *Eco*O109I and *Rsa* I. *Rsa* I cuts only red-specific exon 5, whereas *Eco*O109I cuts both the C allele of red and the green exon 5 sequences. Fragments were separated on a 6% polyacrylamide gel. Lane designations are as in *B*.

DISCUSSION

We observed that whenever more than one green opsin gene is present in the X chromosome-linked red–green gene array, only one is expressed into mRNA in the retina. Individuals known to have both the A and C alleles of exon 5 in their genomic DNA were found to always express only the A allele. This finding demonstrates selective gene expression in an array of homologous genes.

A frequent explanation for nonexpression of genes in a multigene family is the gradual accumulation of enough mutations to block gene function or the phenomenon of pseudogenes. However, it is unlikely that the unexpressed C alleles represent such pseudogenes. First, the C allele was expressed in the retina of individual CV2819, who had only the one green opsin gene (Table 2; Fig. 2). Second, since the majority of the population has more than one green opsin gene, there would be a very high frequency of the putative green opsin pseudogenes. Considering the high frequency of recombination between opsin genes (8, 9), a relatively large proportion of males would be expected to have deutan color vision deficiencies associated with grossly normal gene arrays consisting of one red opsin gene but only green opsin pseudogenes. This is not what has been observed. Deutan color vision

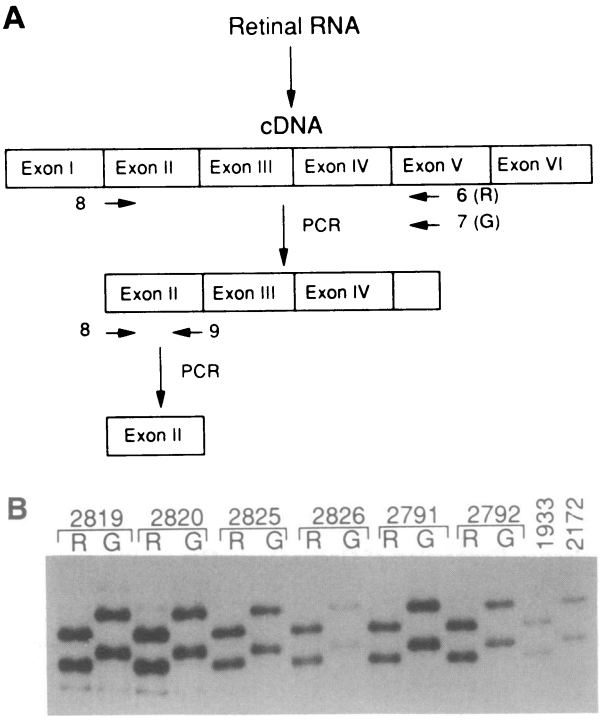

FIG. 3. Sequence analysis of exon 2 of red and green opsin cDNA. (*A*) PCR amplification strategy. Primers are indicated with arrows (see Table 1 for sequence). Arrows G or R are specific for green or red sequences, respectively. (*B*) Autoradiograph of a SSCP gel showing single strands of red-specific (R) lanes and green-specific (G) lanes of exon 2 DNA fragments amplified by using retinal cDNA as a template. Genomic DNA of color-deficient subject 1933, who had only a red opsin gene, and of subject 2172, who had only a green opsin exon 2 sequence (5), was used as a template to amplify reference red and green exon 2 sequences. Identification numbers are given above lanes.

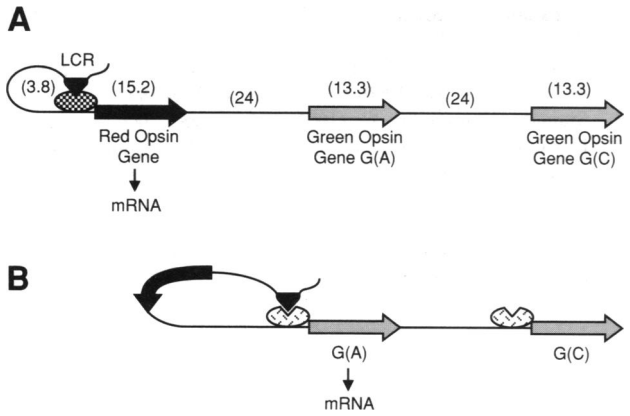

FIG. 4. Model proposed for selective transcription based on position of green opsin gene in the gene array. Numbers between brackets represent length (in kb) of red and green opsin genes and the intervening sequences as described (1–3, 6). (*A*) In the red-sensitive cones, where red gene-specific transcription factors are present, the LCR switches on transcription from the red opsin gene. (*B*) In the green-sensitive cones, where green gene-specific transcription factors are found, the LCR allows transcription of only the closest green opsin gene. The other downstream green opsin genes do not interact with the LCR and are not transcribed.

defects in 58 Caucasian males were shown to be associated either with the deletion of green opsin genes (19 subjects), with the presence of 5′ green–red hybrid genes (38 subjects), or with the C203R point mutation (1 subject) (4, 5, 7).

To explain the selective expression of a green opsin gene, we propose that a locus control-like region (LCR) regulates expression of the genes in the array in a position-dependent manner (Fig. 4). Evidence for such a LCR at the red/green opsin locus has been provided by Nathans *et al.* (6), who studied the molecular basis of X chromosome-linked loss of both red and green pigment function, a condition known as blue cone monochromacy. In 8 of 12 families, they showed a deletion of regulatory sequences located 3.8–4.3 kb upstream of the transcription initiation site of the red opsin gene and 43 kb upstream of the transcription initiation site of the first green opsin gene.

The concept of the locus control region was first suggested for the human β-like globin gene cluster as the upstream sequence element essential for correct developmental switching of the expression from ε- to γ- to β-globin genes (18–20). It was shown recently that correct timing of γ- and β-globin gene activation depended on the relative distance of the γ- and β-globin genes to the LCR, implicating gene order as an important factor. This observation led to a model in which the more proximally located genes have competitive advantage to interact with the LCR (21).

Similar to the findings in the β-like globin gene cluster, we suggest gene order to be important in the expression of green opsin genes in an array in that the most proximal green opsin gene is most likely to be expressed. Possibly, the organization of chromatin (22) at the red/green opsin locus may play an important role in selective expression.

According to the proposed model, the preferential expression of the A allele reflects nonrandom distribution of this allele in the array. It is most likely that the A allele occupies the most proximal position—i.e., the position just downstream of the red opsin gene as shown in Fig. 4.

The observation that all green-sensitive cones express only one green opsin gene explains some of the features of genotype–phenotype relationships in color vision. Nathans *et al.* (4) and Piantanida (23) observed that the relative red/green sensitivity of color-normal observers is not correlated with the number of green opsin genes in the array, as would be expected when only one green opsin gene is expressed. We observed that the frequency of 5′ green–red hybrid genes is higher than the frequency of phenotypic color vision defects among Caucasians and in particular among Afro-Americans (8, 9). Recently, we demonstrated unequivocally that 4 of 129 Caucasian males with anomaloscopically proven normal color vision had a 5′ green–red hybrid gene in addition to normal red and green pigment genes (5). We also found an individual with normal color vision who had a point mutation expected to cause a color vision defect (C203R) in only one of his five green pigment genes (7). These findings could be explained by assuming that in such individuals the 5′ green–red hybrid genes (or the mutant green pigment genes) are located downstream of a normal green pigment gene in the array and are not expressed. A deutan type of color vision deficiency would only be expected when the hybrid (or mutant) gene occupies a position just downstream of the red pigment gene and is expressed. Our data provide information regarding gene expression in a multigene family and demonstrate expression of a single upstream gene among multiple genes in an array.

We also observed that retinal tissue from all 13 subjects contains only a single molecular species of mRNA encoding red opsin and no green–red hybrid gene transcripts. Together with the observation that a single green opsin gene is expressed in the retina, these results do not support the proposal that normal color vision involves the interaction of alleles of two red and two green opsin genes per X chromosome-linked gene array (16, 17). Our findings support the scheme proposed by Nathans *et al.* (4) in which a red–green gene array of most individuals with normal color vision is composed of a single red opsin gene 5′ upstream of one or more green opsin genes.

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