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Number and Variations of the Red and Green Visual Pigment Genes in Japanese Men with Normal Color Vision

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Purpose: We analyzed the red/green visual pigment genes in color-normal Japanese men to understand the relationship between color anomalies and genetic defects.

Methods: DNA from 120 color-normal Japanese men was subjected to polymerase chain reaction (PCR)-amplification for exons 2–5 of the red/green visual pigment genes and the PCR products were sequenced. The red:green gene ratios were estimated from the sequencing electropherograms of exon 5 and also from *Mva*I-restriction fragment analysis of the same exon. The first gene and the downstream genes in the pigment gene array were separately analyzed by PCR, direct sequencing, and/or single-strand conformation polymorphisms.

Results: The red:green gene ratios estimated from the ratios of peak heights of nucleotides on the sequencing electropherograms coincided with those estimated from the *Mva*I-restriction fragment analysis. Among the subjects analyzed, they were 1:1 in 43% (n = 52), 1:2 in 41% (n = 49), 1:3 in 6% (n = 7), and 1:>3 in 9% (n = 11). The first gene in the pigment gene arrays was red in all subjects. Only 1 subject (N22) had a green-red hybrid gene. Exons 2 and 4 had 2 haplotypes each, but exon 3 was highly polymorphic. Exon 5 of the green genes had one polymorphism at codon 283 with a frequency of 32%.

Conclusions: The features of visual pigment genes in color-normal Japanese men were revealed. The data and establishing techniques may be useful for analyzing these genes in color-deficient subjects in the Japanese population. **Jpn J Ophthalmol 2001;45:60–67** © 2001 Japanese Ophthalmological Society

Key Words: Color vision, cone pigment, Japanese, visual pigment genes, X-linked.

Introduction

Visual pigment genes were identified in 1986 by Nathans et al.[1] Among them, the blue pigment gene is present in human chromosome 7, while the red/green genes are arranged in a head-to-tail tandem manner on the X chromosome and are similar to each other; two of the 6 exons (1 and 6) are identical and the other exons differ by only 24 of 872 nucleotides (3%). Although more than half the arrays contain one red and multiple green genes, [2-4] it was hypothesized that only the first two genes in the array are expressed.[5,6]

Deeb et al[6] reported that protan color-vision defects are associated with the presence of a red-green hybrid gene, which occupies the first position in the array but has the green exon 5, and that deutan color-vision defects are associated with green gene deletions or with the presence of a green-red hybrid gene(s) (the term "green-red hybrid gene" means that the gene has the red exon 5 but it is present at positions other than the first in the array). Several studies[3,4,6] have reported the presence of green-red hybrid genes in color-normal white men.

Although Jørgensen et al[7] and Deeb et al[8] studied Japanese men, no color vision tests were performed, and therefore, no data about color-normal Japanese men were obtained. In order to understand the relationship between color anomalies and defects in the

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visual pigment genes in Japanese subjects, it is necessary to analyze the genes in color-normal controls. Therefore, in this study, we determined ratios, numbers, and nucleotide sequences (exons 2–5) of the red/green pigment genes in 120 color-normal Japanese men by polymerase chain reaction (PCR), direct sequencing, restriction analysis, and/or single-strand conformation polymorphisms (SSCP).

Materials and Methods

Human Subjects

DNA was isolated from 120 volunteer Japanese men with normal color vision, which was assessed with Ishihara pseudoisochromatic plates, and, if the subject misread one plate or more, with a Nagel anomaloscope (Schmidt & Haensch, model I, Berlin, Germany). All volunteers were informed of the aims and methods of this study and gave their consent. All procedures were performed in accordance with the Declaration of Helsinki. Usually, 25–50 μg of DNA was obtained from 2 mL of peripheral blood.

Analysis of Visual Pigment Genes

Exons 2, 3, 4, and 5 of the red/green visual pigment genes were PCR-amplified, as depicted in Figure 1. Primer sequences are listed in Table 1. Exons 2 and 5 were amplified with primer pairs 11/12 and 17/16, respectively, in a single PCR step for both red and green genes. The PCR parameters for exon 2 were 94°C (30 seconds)–68°C (30 seconds)–72°C (30 seconds) for 35 cycles, and those for exon 5 were 94°C (30 seconds)–61°C (30 seconds)–72°C (30 seconds) for 35 cycles. Exons 3 and 4 were subjected to a two-step PCR process to amplify either the red-type or the green-type exon. Primer pairs 1/7 or 1/8 were used to amplify the sequence upstream of a red-type or green-type exon 4, respectively. A second PCR with primers 1/2 was then used to amplify exon 3. Primer pairs 3/9 or 3/10 were used to amplify the sequence upstream of a red-type or green-type exon 5, respectively. A second PCR with primers 3/4 was then used to amplify exon 4. The PCR parameters for the first reaction were 94°C (30 seconds)–68°C (30 seconds)–72°C (1 minute) for 20 cycles, and those for the second reaction were 94°C (30 seconds)–61°C (30 seconds)–72°C (30 seconds) for 25 cycles. Takara *Taq* DNA polymerase (Takara, Kyoto) was used. The PCR products were separated on a 2% agarose gel. DNA was purified from the gel with the BandPrep DNA extraction kit (Pharmacia, Uppsala, Sweden). About 20 ng of the purified DNA was used for sequence determination with the dRhodamine terminator cycle sequencing ready reaction kit (PE Applied Biosystems, Foster City, CA, USA) and the sense primers in PRISM 310 Genetic Analyzer (PE Applied Biosystems).

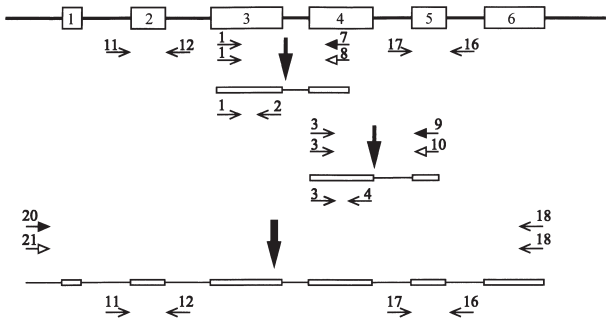

Figure 1. Strategies for polymerase chain reaction (PCR)-amplification. Boxes represent exons, number of which is shown inside. Thick arrows indicate that two-step PCR was carried out, gene-specifically first and in nonspecific way second; amplified segments by first PCR are shown below each thick arrow. Arrows with thin arrowheads (→ and ←) are common primer to both red/green genes and other arrows are gene-specific primers (see Table 1).

Ratio of Visual Pigment Genes

The ratios of the red/green genes were estimated based on the peak heights of nucleotides on the sequencing electropherograms. Standard templates were prepared as follows. The PCR-amplified red- or green-type exon 5 from color-deficient subjects was subcloned into the pUC119 vector. The recombinant plas-

Table 1. Polymerase Chain Reaction Primers Used in This Study

Primer	Position	Sequence (5′ to 3′)
20	−819 –802*	GAGGCGAGGCTACGGAGT
21	−424 –403†	TTAGTCAGGCTGGTCGGGAACT
18	exon 6	GCAGTGAAAGCCTCTGTGACT
11	intron 1	CGGTGCTGCAGCCCAGCTCC
12	intron 2	CGAGCCTGGGCCCCGACTGGC
1	exon 3	GGATCACAGGTCTCTGGTCTC
2	exon 3	CTGCTCCAACCAAAGATGG
7	exon 4 (red)	CATGATGATAGCGAGTGGGATG
8	exon 4 (green)	CACGATGATGCTGAGTGGGGT
3	exon 4	CCACGGCCTGAAGACTTCATGC
4	exon 4	CGCTCGGATGGCCAGCCA
9	exon 5 (red)	GCAGTACGAAAGATCATCACC
10	exon 5 (green)	AGCAGAATGCCAGGACCATC
17	intron 4	TCCAACCCCCGACTCACTATC
16	intron 5	ACGGTATTTTGAGTGGGATCTGCT

*First gene promoter.
†Downstream gene promoter.

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mids were cut with *Eco*RI, the concentration adjusted to 3×10^{-15} g/μL, and used as the template for the PCR amplification of exon 5 at 7 different red:green ratios (4:1–1:4). After electrophoresis, the products were purified from the gel and sequenced. These procedures (PCR-sequencing) were repeated eight times.

The ratios of the red/green genes were also estimated from the amounts of restriction DNA fragments of exon 5. The PCR-amplified exons 5 were digested with *Mva*I (an isoschizomer of *Eco*RII, Takara) and loaded to a 6% polyacrylamide gel. The red exon 5 can be cut at one site with the enzyme (at codons 295-296, CCTGG) to yield 173 bp- and 141 bp-DNA fragments, but in the green exon 5 the 173 bp fragment can be further cut into 112 bp- and 61 bp-fragments (at codons 274–276, CCTGG; red exon 5 is not cleavable there due to the different sequence, CTTTG). The molecular weight marker was the Ready-Load 100 bp DNA ladder (Gibco-BRL Life Technologies, Rockville, MD, USA). After electrophoresis, the gel was stained with SYBRGold (Molecular Probes, Eugene, OR, USA). DNA bands were visualized in the Epilight UV detector (EU-1100, Aisin Cosmos R&D, Tokyo) and their fluorescence intensities were measured by Luminous Imager (Aisin Cosmos R&D). The intensity of the 112 bp-band (= green) was multiplied by 1.54 (= 173/112) and divided by that of the 173 bp-band (= red). These procedures (PCR-restriction cut) were repeated at least three times.

Polymorphisms at Codon 283 in Green Gene

The number of green genes having a C-nucleotide at codon 283 was estimated from peak-heights of the nucleotides (A and/or C) on the sequencing electropherograms as follows. If no signal for "A" is detected, all of the green genes are considered to have "C" at the codon. If the ratio of red and green genes is 1:2 and, nevertheless, the ratio of the peak-heights at the codon indicates C:A to be 2:1, then one of the two green genes is judged to have "C" and the other to have "A." Moreover, if the ratio of the red and green genes is 1:3, and the ratio of the peak-heights indicates C:A to be 1:1 (= 2:2), then one of the green genes is presumed to have "C" and the other two to have "A" at codon 283. These interpretations were verified by SSCP analysis of the downstream genes. We did not count genes in which the exon order was uncertain or in the subjects with more than 3 green genes.

Analysis of the First Gene

Because exons 2 and 5 were independently amplified from both red/green genes, their linkages in vivo had to be determined. For this purpose, exons 2 and 5 of the first gene in the pigment gene array were analyzed. An initial PCR was performed using *Ex*Taq DNA polymerase (Takara) with primers 20 and 18 (Table 1, Figure 1); primer 20 is specific for the promotor of the first gene. The PCR parameters were 98°C (10 seconds)–68°C (15 minutes) for 20 cycles. An aliquot (1/20) of the first reaction mixture was used as the template in additional reactions to amplify exon 2, using primers 11 and 12, and exon 5 using primers 17 and 16. The PCR parameters for exon 2 were 94°C (30 seconds)–68°C (30 seconds)–72°C (30 seconds) for 25 cycles, and those for exon 5 were 94°C (30 seconds)–61°C (30 seconds)–72°C (30 seconds) for 25 cycles. When needed, exons 3 and/or 4 were also amplified. The products were subjected to sequencing. The products from exon 5 were also analyzed by SSCP (the procedures are described in the next section).

Analysis of the Downstream Genes

We amplified the downstream genes (ie, those following the first gene) and analyzed each exon 5 by SSCP to determine whether it contained a red-type exon 5. An initial PCR was performed using *Ex*Taq DNA polymerase with primers 21 and 18 (Table 1, Figure 1); primer 21 is specific for the promotor of downstream genes in the pigment gene array. The PCR parameters were 98°C (10 seconds)–68°C (15 minutes) for 20 cycles. An aliquot (1/20) of the first reaction mixture was used as the template in an additional reaction to amplify exon 5, using primers 17 and 16. The PCR parameters were 94°C (30 seconds)–61°C (30 seconds)–72°C (30 seconds) for 25 cycles. A small portion of the amplified products was mixed with a denaturing solution containing 80% formamide and 20 mM EDTA (pH 8). The mixture was boiled, rapidly chilled, and then applied to a 10% polyacrylamide gel. The electrophoresis was carried out at 220 V for 1.5 hours. The gel was silver-stained as described by Merril et al.[9]

Standard exon 5 used in the SSCP analysis was PCR-amplified from color-deficient subjects who have either red, variant red (codon 296 = C), green, or variant green (codon 283 = C) exon 5. The variant red gene was reported to be present at a frequency of 9% in color-normal white men.[10]

Results and Discussion

Ratio of Visual Pigment Genes

The ratio of the red and green pigment genes has been investigated previously. [2–7,11] They employed

Southern hybridization using radiolabeled probes and/or PCR—^{32}P-endlabeling—restriction digestion, followed by densitometric analyses of detected bands on autoradiograms. The Southern hybridization is the most logical method and has been widely used,[2,4,5–7,11] but, as the original developer mentioned,[11] the transfer- and hybridization-efficiencies are sometimes different among DNA fragments. In the PCR method it is difficult to determine whether the PCR products faithfully keep the original ratio in the templates. Therefore, both methods involve some uncertainty in the estimation of gene ratios. In fact, in the comparison by Wolf et al,[4] the ratios estimated by these methods coincided with each other in only 48% (12/25 samples). These results suggest the necessity for inclusion of standard DNAs (or templates) in the estimation. We intended to avoid the usage of radioisotopes in this study.

Table 2 (column S) shows typical ratios of peak heights of nucleotides on the electropherograms when the PCR products from standard red and green templates (exon 5, 1:1) were sequenced. Figure 2 shows how these peak-height ratios changed as the template ratios varied from 4:1 to 1:4 (red:green). we estimated ratios of the red and green pigment genes based on these standards. Although it may be claimed that the dye terminator method we employed in this study may not be suitable for such a quantitative purpose, we obtained consistent results, as indicated by their averages and standard deviations (Table 2, in parentheses) from at least eight separate analyses of representative samples from each category.

We also estimated ratios of the red/green pigment genes from *Mva*I-restriction fragment analysis of PCR-amplified exon 5. Figure 3A shows typical results of gel electrophoresis when the PCR products from the standard red and green templates (red:green = 2:1–1:4) or five representative samples were cut with *Mva*I. Another restriction endonuclease, *Rsa*I, has been used to discriminate between red exon 5 and the green one,[3,4] but the enzyme cuts red exon 5 only. If the digestion by the enzyme is incomplete, the results must be unstable and unreliable. Since *Mva*I cuts exon 5 at one site common to both exons 5 (at codons 295-296, CCTGG), and at an additional site in the green one (at codons 274-276, CTTTG in red exon 5, with CCTGG in green exon 5) we can easily monitor whether the enzyme worked well or not. Figure 3B shows the ratio of fluorescence intensities (green/red) when the standard templates (red:green = 2:1–1:4) were used, and Figure 3C shows the distribution of the ratios of fluorescence intensities (green/red) in the 120 subjects studied.

The ratios estimated from the sequencing method and those from the *Mva*I-restriction fragment analysis method coincided with each other. The red:green gene ratios and the percentage of subjects we found with each ratio are as follows: 1:1, 43% (n = 52); 1:2, 41% (n = 49); 1:3, 6% (n = 7); and 1:>3, 9% (n =

Table 2. Peak-Height Ratios and Gene Ratios*

	Nucleotide			Gene Ratio				
Codon	Red	Green	S	1 (n=52)	2 (n=49)	3 (n=7)	>3 (n=11)	N22
275-1	T	C	0.9	0.9±0.1	2.3±0.4	3.0±0.5	5.5±1.1	0.5
				(1.0±0.2)	(2.2±0.6)	(3.5±1.1)	(5.4±1.5)	±0.1
-3	T	G	0.9	1.0±0.2	2.2±0.4	3.5±0.9	5.5±0.9	0.5
				(1.2±0.2)	(2.4±0.4)	(3.4±0.9)	(5.7±1.3)	±0.1
277	A	T	1.8	2.1±0.4	4.4±0.9	7.1±1.7	11.5±1.8	0.8
				(1.9±0.3)	(4.7±0.9)	(7.6±1.3)	(12.4±3.5)	±0.2
279	G	T	1.4	1.7±0.2	3.9±1.0	5.6±1.1	10.3±2.0	0.7
				(1.8±0.3)	(4.0±0.6)	(6.0±1.2)	(9.6±1.7)	±0.1
285	A	G	0.3	0.3±0.1	0.8±0.1	1.2±0.3	1.9±0.3	0.2
				(0.4±0.1)	(0.9±0.1)	(1.3±0.2)	(2.1±0.5)	±0.1
309	A	T	1.3	1.4±0.2	2.9±0.5	4.0±0.8	7.6±1.7	0.6
				(1.3±0.2)	(2.9±0.2)	(4.3±0.7)	(7.9±2.1)	±0.1

*Values are ratios of peak heights of nucleotides on sequencing electropherograms. S: Standard ratios when authentic red and green templates (1:1) were polymerase chain reaction (PCR)-amplified for exon 5 and sequenced. Averages and standard deviations are presented separately for groups 1, 2, 3, and >3 where ratio of red:green genes was estimated to be 1:1, 1:2, 1:3, 1:>3, respectively. DNA from representatives of each group (one each) was used to PCR-amplify and sequence exon 5 at least eight separate times; averages and standard deviations are shown in parentheses. Same determinations were repeated for N22. Other 5 codons where red and green genes differ were not useful for estimation of gene ratios because of high background, small peaks, extreme unbalance between peak heights of two nucleotides, or presence of polymorphism.

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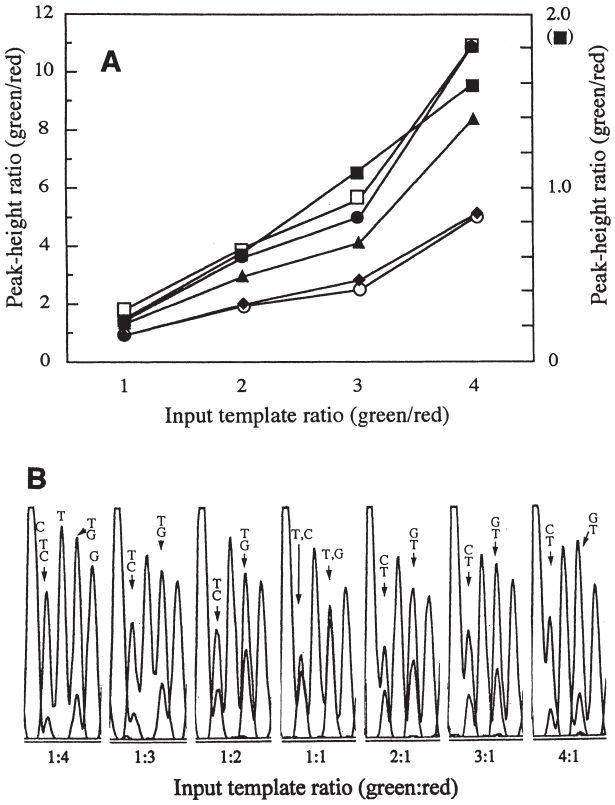

Figure 2. Relationship of template ratios and peak-height ratios. (**A**) Exon 5 was polymerase chain reaction (PCR)-amplified from cloned templates (green:red = 1:1–4:1) and sequenced. Peak heights of nucleotides on sequencing electropherograms were compared. –○–: codon 275-1, –◆–: codon 275-3, –□–: codon 277, –●–: codon 279, –■–: codon 285, –▲–: codon 309. Values are mean of eight independent determinations, but standard deviations are not presented to avoid complexity; they were similar to values shown in Table 2. (**B**) Sequencing electropherograms (around codon 275) of PCR products at seven different template ratios.

11). Our results are somewhat different from those reported by Jørgensen et al[7] and Deeb et al[8] who analyzed the visual pigment gene arrays in Japanese men, but did not perform color vision tests; in these previous reports the red:green gene ratios and the

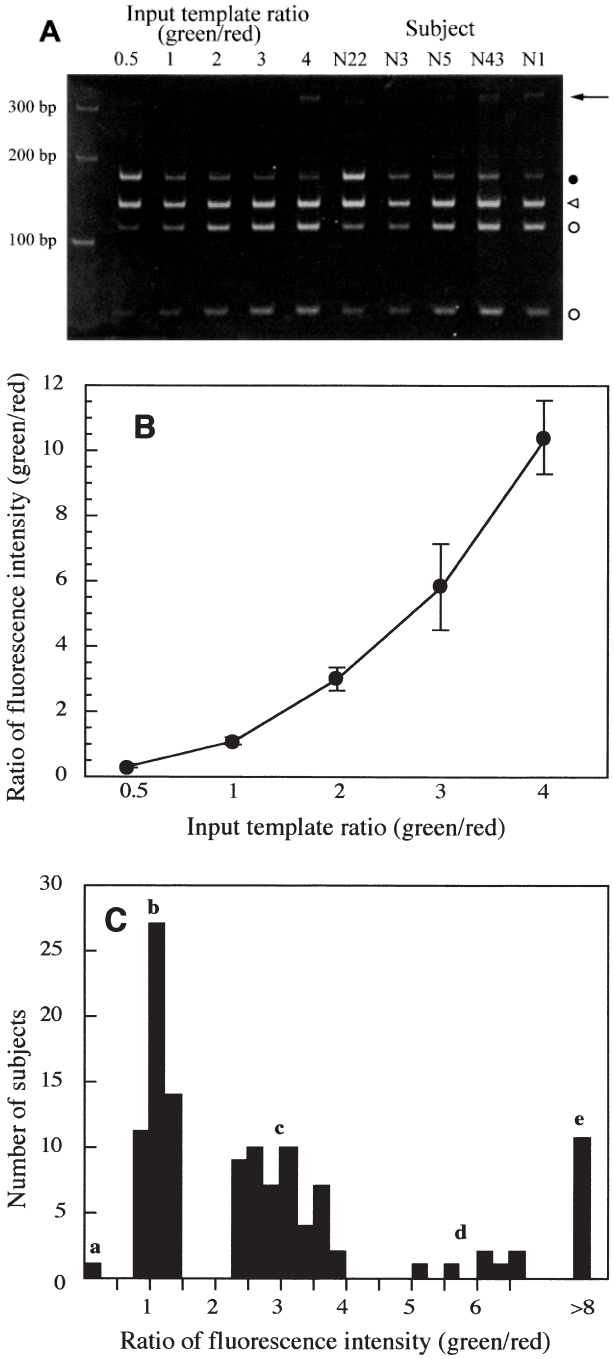

Figure 3. Estimation of gene ratios from restriction fragment analysis. (**A**) Typical electrophoretic pattern of *Mva*I-digested exon 5. Exon 5 was amplified from standard templates (red:green = 2:1–1:4) or from five representative DNA samples, and products were loaded to a 2% agarose gel. After electrophoresis, 314 bp-DNA fragments were extracted from gel. Purified DNA was digested with *Mva*I, and loaded to a 6% polyacrylamide gel. After electrophoresis gel was stained with SYBRGold. ←: position of 314 bp product, ●: red-specific band (173 bp), ○: green-specific band (112 bp and 61 bp), ◁: common band to both red and green exons 5. Estimated gene ratios (red:green) were 2:1 in N22, 1:1 in N3, 1:2 in N5, 1:3 in N43 and 1:>3 in N1. (**B**) Standard curve for estimation of gene ratios. Ratios of fluorescence intensities (green/red) obtained from standard templates are presented as means (●) and standard deviations (bars) of 6 separate determinations. (**C**) Distribution of ratios of fluorescence intensities (green/red) in 120 subjects studied. Each value the mean of 3–6 determinations. Estimated gene ratios (red:green) are 2:1, 1:1, 1:2, 1:3 and 1:>3 for groups a, b, c, d, and e, respectively.

percentage of subjects found with each ratio are as follows: 1:1, 49%; 1:2, 30%; and 1:>2, 21%. Based on these data, it may be concluded that about 80% of color-normal Japanese men have 1:1 or 1:2 gene ratios (red:green) and the rest have higher gene ratios.

Because our data were obtained solely from analysis of the PCR products, they should be examined by a different and more reliable method, which is so far not available.

Number of Visual Pigment Genes

As the analysis of exon 5 gave just the ratio of the green/red genes, the total number of these genes should be determined. Neitz and Neitz[3] and Wolf et al[4] amplified promoters of the red/green genes by PCR and analyzed them by restriction digestion, but the method seems to sometimes give double numbers [4,12] for unknown reasons. Macke and Nathans[13] used pulsed-field gel electrophoresis and Southern blotting, and Wolf et al[14] developed a direct visualization technique to determine gene copy number. These are reliable methods, but not easy to follow. Yamaguchi et al[12] amplified promoters and analyzed them by SSCP; the method is convenient and inexpensive, but it requires handling of radioisotope-labeled DNA fragments, which we intended to avoid in this study.

With consideration of these previously described methods, we took a different approach. First, we analyzed the first gene in the pigment gene array by SSCP analysis. They were all red genes; typical profiles are shown in Figure 4. Then we analyzed the downstream genes to examine whether they contain a hybrid-red gene(s) or not. All the subjects except one (N22), had only green genes (typical and/or variant) downstream. Typical profiles are shown in Figure 4; N22 shows the presence of a red exon 5 in the downstream genes. These results indicate that most color-normal Japanese men have only one red gene. We have also examined promoters of the green/red genes, and reached the same conclusion (Ueyama et al, Color Res Appl, in press). Thus, the occurrence of a green-red hybrid gene is quite rare (<1%) in Japanese. In the white population, however, the presence of such a gene is more common, reported to be >4%[6] or >8%[15].

N22 was suggested from the promoter analysis to have 3 visual pigment genes (data not shown), the content of which should be one red, one green, and one green-red hybrid genes (Table 2). The subject had normal color vision as assessed not only by Ishihara plates, but also by an anomaloscope (Rayleigh's match). We had already confirmed that the first gene in the array was red (Figure 4). In some color-normal white men who have a green-red hybrid gene(s), the order of genes has recently been determined to be red, green and then green-red hybrid.[16]

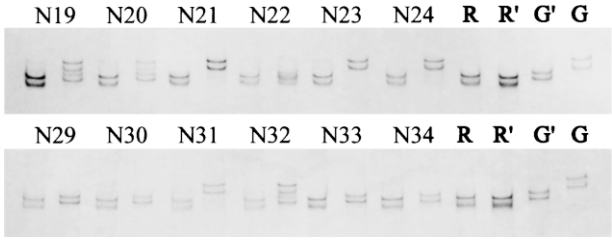

Figure 4: Single-strand conformation polymorphism (SSCP) analysis of pigment genes. Typical SSCP profiles of exons 5 of first gene (left) and of downstream genes (right) are presented as pairs. The markers for exon 5: R: typical red, R′: variant red (codon 296 = C), G′: variant green (codon 283 = C), G, typical green. All subjects except N22 had green exon(s) 5 downstream, but not typical red or variant red exon 5 there; only N22 had red and variant green exons 5 downstream. Variant green exon 5 was also observed in N19, N20, N29, N30, N32, N33, and N34.

Sequence Diversity of Visual Pigment Genes

In this section, lower-case letters are used to indicate nucleotide polymorphisms without accompanying amino acid substitution, and upper-case letters to indicate those with accompanying amino acid substitutions. Exons 2 and 4 had two haplotypes each. In exon 2, codons 65, 100, 111 and 116 were either C, a, A and C, respectively, or T, g, G, and A, respectively. We termed the former the red-type and the latter the green-type. Likewise in exon 4, codons 230, 231-1, -2, -3, and 236 were T, G, C, T, and A, respectively, or C, A, G, C, and G, respectively. We termed the former the red-type and the latter the green-type. The red-type exons were occasionally found in the green genes (5/158), and the green-type exons were sometimes found in the red genes (5/119).

Tables 3 and 4 summarize polymorphisms and haplotypes of exons 3 and 5. As for the first 3 polymorphic sites in exon 3, ie, codons 151, 153, and 155, 65% (77/119) of the red genes were g, C and g, respectively, and fewer (34%; 40/119) were a, A, and c, respectively (Table 3). A reversed relationship existed in the green genes; 77% (122/158) were a, A, and c, respectively, and only 6% (9/158) were g, C, and g, respectively (Table 4). From these results we call the former the green-type and the latter the red-type. One of the fused types, 5′ red-green (g, C, and c) was observed only in the red genes and the other,

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Table 3. Polymorphisms and Haplotypes of Red Genes*

Polymorphic codon (exon 3)							Haplotype Frequency	
151	153	155	171	174	178	180	%	(n)
g	C	g	G G	C	A	T	53	(63)
g	C	g	A T	C	A	T	2	(2)†
a	A	c	G G	C	A	T	20	(24)
g	C	g	G G	C	G	T	2	(2)†
a	A	c	G G	T	A	T	1	(1)†
g	C	c	G G	C	A	T	2	(2)‡
a	A	c	G G	C	A	G	12	(14)
g	C	g	G G	C	A	G	8	(9)
g	C	g	A T	C	A	G	1	(1)‡
a	A	c	G G	T	G	G	1	(1)*

*Total number of red genes is 119, because no data for exons 3 and 4 was obtained in N91. Green-red hybrid gene in N22 was not included in this table. Nucleotides at codons 283 and 296 (both in exon 5) were C and T, respectively, in all subjects. Lower-case letters indicate polymorphisms without accompanying amino acid substitution.

†Haplotypes that have not been reported in Japanese.

‡Haplotypes that have not been reported before.

Table 4. Polymorphisms and Haplotypes of Green Genes*

Polymorphic Codon								Haplotype Frequency	
Exon 3							Exon 5		
151	153	155	171	174	178	180	283	%	(n)
a	A	c	G G	C	A	G	a	45	(71)
							c	25	(40)
g	C	g	G G	C	A	G	a	1	(2)
							c	1	(1)†
a	A	g	G G	C	A	G	a	3	(5)‡
							c	3	(4)
a	A	g	A T	C	A	G	a	1	(1)‡
a	A	g	G G	T	G	G	a	10	(16)
a	A	c	G G	T	G	G	a	1	(2)†
							c	1	(1)†
g	C	g	G G	T	G	G	a	1	(1)†
a	A	g	G G	C	G	G	a	1	(1)‡
a	A	c	G G	C	A	T	a	4	(7)
							c	1	(1)†
g	C	g	G G	C	A	T	a	1	(1)
							c	2	(3)†
g	C	g	G G	T	A	T	a	1	(1)‡

*Total number of green genes is 158 and number of green genes having C-nucleotide at codon 283 is 50. Lower-case letters indicate polymorphisms without accompanying amino acid substitution.

†Haplotypes that have not been reported in Japanese.

‡Haplotypes that have not been reported before.

5′ green-red (a, A, and g), was observed only in the green genes.

As for codon 180, "T (Ser)" was found in the majority of the red genes (79%; 94/119) as was "G (Ala)" in the green genes (92%; 145/158). In white subjects the number of the red genes having "T (Ser)" at codon 180 was less (62%).[10]

In both genes, codons 174 and 178 were mainly C and A: 97% (115/119) in the red and 86% (136/158) in the green genes. Variations such as C and G, T and G, or T and A at these codons were also found, at a much lower frequency, in both types of genes.

Deeb et al[8] reported only 5 haplotypes in the red genes in 50 Japanese men (Nisei and Sansei). They did not detect polymorphisms at codons 171, 174, or 178, but we found variations at these codons in 3, 2, and 3 genes, respectively. Novel haplotypes which have not been reported before[10,17] or have not been reported in Japanese[8] were also found (‡ and †, respectively, in Tables 3 and 4).

With regard to exon 5, no polymorphism was present in the red genes as revealed by analyses of the first genes. The nucleotide at codon 296 was "t" in all subjects, in contrast with a high (9%) frequency of "c" in white subjects.[10] In the green genes, however, one polymorphism was detected, ie, at codon 283 ("a": 68% and "c": 32%, Table 4). Deeb et al[8] also analyzed the green pigment genes in Japanese men. One major difference between our data and theirs is in the frequency of this polymorphism; they detected C-nucleotide at codon 283 in only 5% of subjects. We do not know the reason for this discrepancy, but it may be ascribed to the difference in detection methods. We applied sequencing to all subjects, while they used SSCP analysis. We examined 52 subjects with only one green gene in addition to one red gene and therefore it was easy to analyze them; 17 had "c" at this codon, suggesting that a 32% occurrence would be correct. Moreover, SSCP analyses of the downstream genes also demonstrated such a high occurrence (Figure 4). It is, however, still lower than that in white subjects (49%).[10]

Conclusions

We identified the features of visual pigment genes in 120 color-normal Japanese men. (1) The red:green gene ratios were 1:1 in 43% (n = 52), 1:2 in 41% (n = 49), 1:3 in 6% (n = 7), and 1:>3 in 9% (n = 11). (2) All had a red gene at the first position in the array. (3) The occurrence of a green-red hybrid gene in color-normal Japanese men is quite rare (1/120). (4) Exons 2 and 4 had only two haplotypes each. (5) Exon 3 was highly polymorphic. (6) Variant green exon 5 (codon 283 = "c") was six times more frequent than previously reported, but still less frequent than in white subjects.

These profiles and the techniques we established in this study may be useful in analyzing these genes in color-deficient subjects, e.g., we have already found a haplotype, consisting of atypical exons 2, 4, and 5, in 3 color-deficient subjects, which is not present in color-normals. This haplotype may be genetically related to color vision defects in the Japanese population. Moreover, we have found 9 color-deficient subjects in whom organization of the pigment genes is the same as in color-normals. The details will be presented elsewhere.

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