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Unique haplotype in exon 3 of cone opsin mRNA affects splicing of its precursor, leading to congenital color vision defect

Hisao Ueyama [a,*], Sanae Muraki-Oda [b], Shinichi Yamade [b], Shoko Tanabe [c], Takahiro Yamashita [d], Yoshinori Shichida [d], Hisakazu Ogita [a]

[a] Department of Molecular Medical Biochemistry, Shiga University of Medical Science, Seta, Otsu 520-2192, Japan
[b] Department of Ophthalmology, Shiga University of Medical Science, Seta, Otsu 520-2192, Japan
[c] Institute of Vision Research, Atsuta-ku, Nagoya 456-0032, Japan
[d] Department of Biophysics, Graduate School of Science, Kyoto University, Kyoto 606-8502, Japan

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ABSTRACT

We have analyzed L/M visual pigment gene arrays in 119 Japanese men with protanopia color vision defect and found that five had a normal gene order of L–M. Among the five men, two (identified as A376 and A642) had apparently normal L genes. To clarify their L gene defect, the whole L or M gene from A376 and control subjects was cloned in an expression vector. Total RNA extracted from the transfected HEK293 cells was analyzed by Northern blot and reverse transcription-polymerase chain reaction. The product from the cloned L gene of A376 was smaller than the normal control due to the absence of exon 3. To investigate such exon-skipping at splicing, minigenes of exon 3 accompanying introns 2 and 3 were prepared from A376, A642, and control subjects. The minigenes of A376 (L) and A642 (L) showed the product lacking exon 3 only, while the minigene of normal control N44 (L) showed the product retaining exon 3 only. Exchanging of introns 2 and 3 between the A376 (L) and N44 (L) minigenes showed that the skipping of exon 3 was caused by the exon itself. Seven differences in exon 3 between A376 (L) and N44 (L) were all within already-known polymorphisms as follows: G^{151-3}, C^{153-1}, G^{155-3}, A^{171-1}, T^{171-3}, G^{178-1} and G^{180-1} in A376 (L) and A642 (L), and A^{151-3}, A^{153-1}, C^{155-3}, G^{171-1}, G^{171-3}, A^{178-1} and T^{180-1} in N44 (L). An *in vitro* mutagenesis experiment with these nucleotides in the minigenes showed that exon 3 was completely skipped at splicing only in the haplotype observed in A376 (L) and A642 (L). These results suggest that complete skipping of exon 3 at splicing, due to the unique haplotype of the exon, causes loss of expression of L-opsin in these men.

1. Introduction

L-cone and M-cone visual pigment genes co-localize in a head-to-tail tandem array on human X chromosome at q28 [1]. The two genes are very similar to each other, with a 98% nucleotide sequence identity in the exons, introns and downstream regions, probably because they arose recently in evolution (30–40 million years ago) through duplication of an ancestral pigment gene [2]. Because of such similarities in structure, unequal recombination between these genes tends to occur [3], resulting in a single L gene-array, a single-L/M hybrid gene array, or an array with a gene order of L–M/L.

The protan color vision defect is characterized by the absence of L-cones in the retina, and has been thought to be caused by a single-L/M hybrid gene array, or by an array with the gene order of L/M–M [4–6]. In the latter case, the color vision phenotype is expected to be protanopia, a color vision status in which no L-cones are functioning, when the products of the L/M and M genes are spectrally identical, or to be protanomaly, a color vision status in which the sensitivity to long-wavelength light is extremely low, when the products are spectrally different. However, in our study of 125 Japanese men with protan defect, seven individuals had an array with the normal L–M gene order [7]. We analyzed L genes from these seven men, and found that two had a missense mutation [7,8], and one subject had a nonsense mutation of Tyr194Stop (later identified), but the other four individuals had apparently normal L genes.

Since our previous study, we have analyzed pigment gene arrays of additional 86 men with protan defect. In the combined total of 211 (125 + 86) men with protan defect, 119 had protanopia and 92 had protanomaly. Among the 119 men with protanopia, five had an array with the normal gene order, two of whom had apparently

Abbreviations: RT, reverse transcription; PCR, polymerase chain reaction.

* Corresponding author. Fax: +81 77 548 2164.
E-mail address: datt@belle.shiga-med.ac.jp (H. Ueyama).

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normal L genes. In the current study, we focused on these two men and intended to clarify the cause of the absence of L-opsin in them. We found that exon 3 was completely skipped at splicing of the primary transcript from their L genes. The skipping of exon 3 was ascribed to a unique haplotype of the exon, and this haplotype was considered to be the cause of protanopia phenotype in these two men.

2. Materials and methods

2.1. Color vision status of the participants, extraction of genomic DNA and analysis of L /M visual pigment gene array

The participants in this study had consulted the Japan Red Cross Nagoya First Hospital or the Shiga University of Medical Science Hospital regarding their color vision. Written informed consent was obtained from all participants and all study procedures were conducted according to the Declaration of Helsinki. This study was approved by the Medical Ethics Committee at the Shiga University of Medical Science (No. 13-7-1). Two hundred and eleven subjects were diagnosed as protan defect by examination with an anomaloscope (Model I, Schmidt and Haensch, Berlin, Germany). Seven male volunteers, whose color-vision status was confirmed to be normal by examination with Ishihara pseudoisochromatic plates, also participated in this study. A blood sample drawn from each participant was used for extraction of genomic DNA.

The most upstream gene and downstream gene(s) of an L/M visual pigment gene array were separately amplified by long-range PCR from genomic DNA as described previously [9]. When the most upstream gene had an L-type exon 5 and downstream gene had an M-type exon 5 only, it was concluded that the participant had an array with the normal gene order.

2.2. Cloning of the L/M opsin gene

The L genes of a color-normal participant (N25) and protan participants (A233, A376, A450 and A769) were amplified as the most upstream gene of the array by long-range PCR. The M gene of another color-normal participant (N219) was also amplified as the downstream gene. These PCR products were used as the template in the second-round long-range PCR for cloning. The primers used were 5′RV (5′-GCGCGATATCGACAGGGCTTTCCATAG-3′, *Eco*RV site is underlined) and 3′Sal (5′-CGGCGTCGACTGCAGGCGATACCGAG G-3′, *Sal*I site is underlined). The PCR product was separated by electrophoresis on a 0.7% agarose gel and then extracted from the gel using the Wizard SV Gel and PCR Clean-Up System (Promega Corp., Madison, WI, USA). The purified product was digested with *Eco*RV (Takara Bio Inc., Otsu, Shiga, Japan) and *Sal*I (Takara Bio Inc.), separated by electrophoresis and the digested product was then extracted from the gel. The purified product was ligated, using Ligation high (Toyobo, Osaka, Japan), with an expression vector, pFLAG-CMV-5a (Sigma–Aldrich, St. Louis, MO, USA). The DH5α *Escherichia coli* cells (Takara Bio Inc.) were transformed with the ligated products. The plasmids harboring the L/M gene were isolated by using NucleoBond PC 100 (Macherey–Nagel, Düren, Germany).

2.3. Preparation of minigenes

The human L gene has five *Bbs*I sites, three in intron 1 and one each in exons 2 and 4. The genomic region of exon 2–exon 4 was amplified by PCR from the genomic DNA of two protanopia participants (A376 and A642) and five color-normal participants (N3, N6, N44, N48 and N102). The primers used were I1F (5′-CGGTGCTG CAGCCCAGCTCC-3′, corresponding to intron 1, −47 to −28 from the 3′-splice site) and I4R (5′-GACTCATTTGAGGGCAGAGCAGC-3′, corresponding to intron 4, +47 to +69 from the 5′-splice site), and the enzyme used was the Tks Gflex DNA polymerase (Takara Bio Inc.). The PCR products were separated by electrophoresis on a 0.7% agarose gel and were then extracted from the gel. The purified products were digested by *Bbs*I (Thermo Fisher Scientific, Waltham, MA, USA) and were separated by gel electrophoresis and then extracted from the gel. The L-opsin cDNA [8] was cloned into the *Kpn*I site of pFLAG-CMV-5a vector, in which both of the *Bbs*I (about 180 bp downstream of the FLAG sequence) and the *Hin*dIII (in the polylinker) sites had been eliminated. The region of the L opsin cDNA from one *Bbs*I site (in exon 2) to the other (in exon 4) was exchanged with the genomic *Bbs*I fragment. The final recombinant plasmids are defined as 'minigenes' in this study.

For the exchange of introns between the minigenes, restriction sites of *Sal*I (in the polylinker) and *Hin*dIII (55 bp upstream of exon 3) were employed for intron 2, and those of *Afl*II (51 bp downstream of exon 3) and *Sfi*I (about 930 bp downstream of the FLAG sequence) were employed for intron 3. *In vitro* mutagenesis of the region between the *Hin*dIII and *Afl*II sites was done using the overlap-extension method as described previously [7,8]. The nucleotide sequence of these minigenes was confirmed by using the BigDye Terminator v1.1 Cycle Sequencing Kit (Life Technologies, Carlsbad, CA, USA) and the ABI Prism 3130*xl* Genetic Analyzer (Life Technologies).

2.4. Transfection and extraction of RNA

HEK293 cells, which were maintained in Dulbecco's modified Eagle's medium (Sigma–Aldrich) supplemented with 10% fetal bovine serum (BioWest, Salt Lake City, UT, USA), were transfected with the gene clones using FuGene HD (Roche, Basel, Switzerland). The cells were collected after a 2 day-incubation, and total RNA was extracted from the cells by using Isogen (Nippon Gene Co., Tokyo, Japan). The isolated RNA was treated with RNase-free DNase (Deoxyribonuclease (RT grade), Nippon Gene Co.) at 37 °C for 15 min, and the DNase was heat-inactivated at 70 °C for 10 min. The final RNA was stored at −80 °C until use.

A human retinoblastoma cell line, WERI-Rb1, which was maintained in RPMI medium (Sigma–Aldrich) supplemented with 10% fetal bovine serum (BioWest), was also used for transfection. This cell line has been confirmed to express L- and M-opsin mRNAs [10,11].

2.5. Northern blot

The RNA samples (5 μg each) were loaded onto a 1.0% agarose gel containing 6% formaldehyde, and RNA size markers (DynaMarker RNA Easy Measurement N; Biodynamics Lab. Inc., Tokyo, Japan) were used.

The separated RNA was blotted to a nitrocellulose membrane (Hybond-C Extra, GE Healthcare Life Sciences, Amersham Place, United Kingdom). The membrane was subjected to hybridization in a solution containing 50% formamide and 0.9 M NaCl at 42 °C overnight. The DNA probe used was the human L-opsin cDNA, which had been labeled with [α-^{32}P]-dCTP (PerkinElmer, Waltham, MA, USA). After hybridization, the membrane was washed finally with a solution containing 30 mM NaCl and 0.1% sodium dodecyl sulfate at 65 °C. The washed membrane was exposed to an X-ray film (RX, Fuji Film, Tokyo, Japan) at −80 °C overnight.

2.6. RT-PCR

RT was carried out for 1 μg of each RNA sample, with SuperScript III RT (Invitrogen, Carlsbad, CA, USA) as the enzyme and Anchored Oligo(dT)$_{20}$ (Invitrogen) as the primer. The reaction conditions were 45 °C for 15 min and then 94 °C for 2 min. After

RT was complete, a portion (4%) of the reaction mixture was used as the template for PCR. The primers used were 5′UTF (5′-GGGACAGGGCTTTCCATAGCC-3′, +21 to +41 in 5′-untranslated region of exon 1) and FLAGR (5′-CTTGTCATCGTCGTCCTTGTA-3′, complementary to the FLAG sequence) for gene clones, and EX2F (5′-TGGATGATCTTTGTGGTCA-3′, corresponding to codons 59–65 in exon 2) and FLAGR for minigenes. The PCR conditions were 94 °C for 2 min and then 25 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, with a final extension at 72 °C for 2 min. The PCR product was analyzed by electrophoresis on a 2.0% agarose gel. The markers were the Gene Ladder Wide 2 (Nippon Gene Co.).

3. Results

3.1. Participants with protan defect having an array with normal gene order

Table 1 presents the details regarding the 11 participants with protan defect having arrays with the normal gene order, seven of whom were described previously [7]. A total of four missense mutations have been found. In the reconstitution experiments of visual pigments, neither of the two new mutations (Pro187Leu and His300Tyr) showed any absorbance, under conditions where wild-type L-cone pigment displayed a spectrum with a λmax of 560 nm (Supplementary Fig. 1).

3.2. Northern blot and RT-PCR analyses

RNA products from the transfected L or M gene, in HEK293 cells, were analyzed by Northern blot and RT-PCR. Three kinds of RNA bands were obtained in the Northern blot (Fig. 1A) and four kinds were seen in the RT-PCR analysis (Fig. 1B). On comparison of these results, it was concluded that bands ‘a’, ‘b’ and ‘c’ in the Northern blot corresponded to bands ‘d’, ‘e’ and ‘f’, respectively in the RT-PCR analysis. Sequencing of each RT-PCR product revealed that band ‘d’ was full-size opsin mRNA, band ‘e’ to that lacking exon 3, and band ‘f’ to that lacking exon 2. Band ‘g’, which was opsin mRNA lacking exons 2 and 3, was visible in the RT-PCR analysis, but it was not detected in the Northern blot. It is noteworthy that the full-size product, band ‘d’, was not visible in the L gene of A376, even by such a highly sensitive method as RT-PCR.

Since the profiles of RT-PCR analysis in transfected WERI-Rb1 cells (Supplementary Fig. 2) were similar to those obtained in HEK293 cells, the elimination of exon 3 was not attributed to HEK293 cells which do not express endogenous opsin mRNAs. Therefore we used only HEK293 cells in the later experiments.

Table 1
Participants with protan color vision defect with an array of normal gene order.

Color vision status	Participants	Match-range[a]	Mutation in L gene
Protanopia	A289	0–73	Pro231Leu (CCA > CTA)
	A318	0–73	Tyr194Stop (TAC > TAG)
	A376	0–73	None
	A557	0–73	Pro187Leu (CCC > CTC)
	A642	0–73	None
Protanomaly	A89	Not done	Gly338Glu (GGG > GAG)
	A185	51–53	None
	A233	58–60	None
	A450	45–62	None
	A769	47.5–52.5	None
	A774	50–55	His300Tyr (CAC > TAC)

[a] Match range is that obtained at examination using an anomaloscope.

3.3. Analysis of splicing pattern using minigenes

To explore the mechanism by which exon 3 is excluded from the precursor of the L-opsin mRNA, we produced cone opsin minigenes from the two participants with protanopia, A376 and A642. We also made minigenes from five color-normal participants, N3, N6, N44, N48 and N102. HEK293 cells were transfected with theses minigenes and their products were analyzed by RT-PCR and gel electrophoresis (Fig. 2A). Minigenes from N3 (M), N3 (L) and N44 (L) yielded only the product retaining exon 3 (lanes 2–4), those from A376 (L) and A642 (L) yielded only the product lacking exon 3 (lanes 8 and 9), and those from N102 (L), N48 (L) and N6 (L) yielded both products (lanes 5–7).

We chose two minigenes, N44 (L)-derived and A376 (L)-derived, for further analysis. Introns 2 and/or 3 were exchanged between these minigenes. Fig. 2B shows that exon 3 was retained whenever the derivation of the exon was N44 (L) and that exon 3 was eliminated whenever the derivation of the exon was A376 (L), indicating that exon 3 itself determines retention/elimination of the exon. Fig. 3 shows seven nucleotide differences in the L gene exon 3 between N44 and A376. The exons 3 of A376 (L) and A642 (L) had identical nucleotide sequences. All seven differences seen here were within already-known polymorphisms [12,13]. We express the haplotype of N44 (L) as ‘AAC/GG/c/A/T’ and that of A376 (L) and A642 (L) as ’GCG/AT/c/G/G’, because the most upstream three nucleotides are linked to each other in most L genes [14], and the next two nucleotides were each in complete linkage disequilibrium [13,14]. The sixth polymorphic site (shown by an asterisk (∗) in Fig. 3) [13,14], where the nucleotide was ‘C’ in common to N44

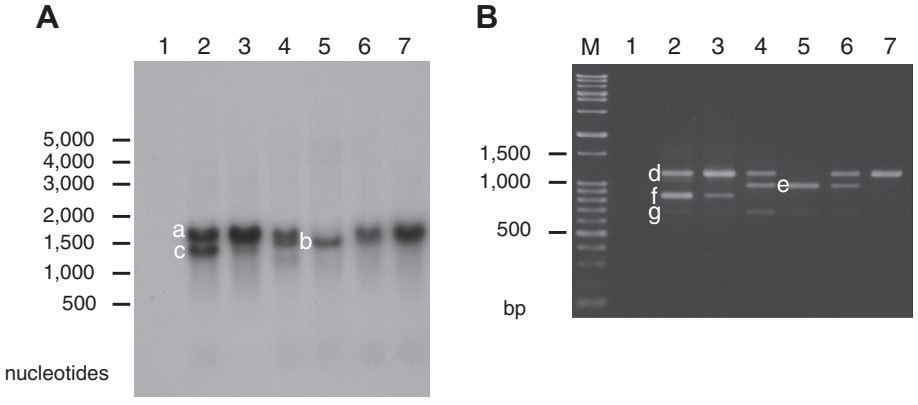

Fig. 1. Northern blot (A) and RT-PCR (B) analyses of products from opsin gene clones. Total RNA was extracted from HEK293 cells, which had been transfected with the cloned L or M opsin gene. The probe used was ^{32}P-labeled L opsin cDNA. 1, No DNA; 2, normal L gene (N25); 3, normal M gene (N219); 4, L gene of A233; 5, L gene of A376; 6, L gene of A450; 7, L gene of A769.

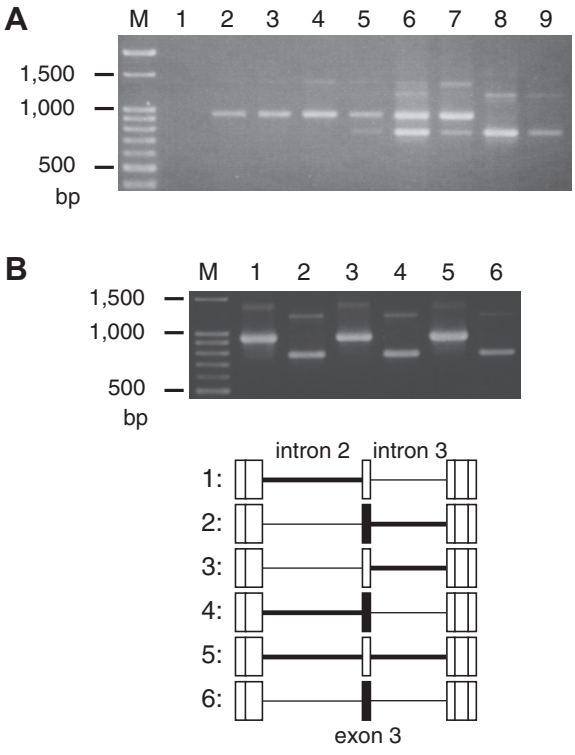

Fig. 2. RT-PCR analysis of the product from minigenes. (A) Lane 1, no DNA; lane 2, N3 (M); lane 3, N3 (L); lane 4, N44 (L); lane 5, N102 (L); lane 6, N48 (L); lane 7, N6 (L); lane 8, A376 (L); lane 9, A642 (L). (B) The drawing shows the structure of minigenes in which intron 2 and/or intron 3 was exchanged between the N44 (L) and A376 (L) minigenes. Exon 3 of N44 (L) is shown by open boxes and that of A376 (L) by closed boxes. Introns 2 and 3 of N44 (L) and A376 (L) are shown by thin and thick lines, respectively. Each number corresponds to that in the photograph above.

(L), A376 (L) and A642 (L), was also included in the haplotype using lower-case letters, c or t.

3.4. In vitro mutagenesis of minigenes, transfection and RT-PCR

In vitro mutagenesis was applied to the N44 (L) and A376 (L) minigenes, and finally a total of 20 different minigenes were obtained. Among these minigenes, only that having the haplotype of A376 (L) and A642 (L) showed a product lacking exon 3 with no product retaining the exon (Fig. 4A). The substitution of the third site 'c' with 't' was found to have some advantageous effect on retaining of exon 3 (Fig. 4B).

The number of haplotypes found in 276 L/M genes of color-normal men [14] and in 182 L/M genes of single-gene dichromats (our cohort) are shown below each haplotype in Fig. 4. The haplotype of 'GCG/AT/c/G/G' was restricted to A376 (L) and A642 (L), and the haplotype of 'GCG/GG/c/G/G' was restricted to A185 (L), A233 (L) and A450 (L) in our cohort study.

4. Discussion

We functionally characterized two missense mutations, Pro187Ser and Val120Met previously reported by others [15,16], as well as two novel missense mutations, Pro187Leu and His300-Tyr. Neither of the reconstituted pigments having these mutations gave any absorbance (Supplementary Fig. 1). In addition to these four mutations, seven missense mutations [7,8,17–19] have been reported in the L/M opsin genes. These mutations would be useful for understanding structure–function relationships in L/M opsins, as in rhodopsin [20]. The nonsense mutation we found (Tyr194-Stop) is the third identified in L/M opsin genes [18,21].

Transfection experiments using the whole opsin gene clone showed that the L gene of A376 yielded an mRNA species lacking exon 3 only. Further experiments revealed that the unique haplotype observed in exon 3 of A376 (L) and A642 (L) caused skipping of the exon at splicing. As a result of the absence of exon 3, a premature termination codon appears in exon 4 (at codon 199). Therefore, functional L opsin is thought not to be produced in these men, leading to the protanopia color vision defect.

The haplotype in exon 3 of the L genes of A376 and A642 was 'GCG/AT/c/G/G', which was not found in a total of 458 normal L/M opsin genes. Therefore, A376 and A642 were revealed to have an extremely rare haplotype in exon 3 of their L genes. The L genes of A185, A233 and A450 were also revealed to have another extremely rare haplotype in common, but the etiological significance for their color vision (protanomaly) is unclear at present.

The very haplotype of A376 (L) and A642 (L) has been reported as the LIAVA combination [15,16,22], which represents Leu^{153}, Ile^{171}, Ala^{174}, Val^{178} and Ala^{180}. Neitz et al. [15] found one subject with deuteranopia, in which no M-cones are functioning, having one each of L and M genes, where the M gene had the LIAVA combination. They also found one subject with protanopia, having one each of L and M genes, where the L gene had the LIAVA combina-

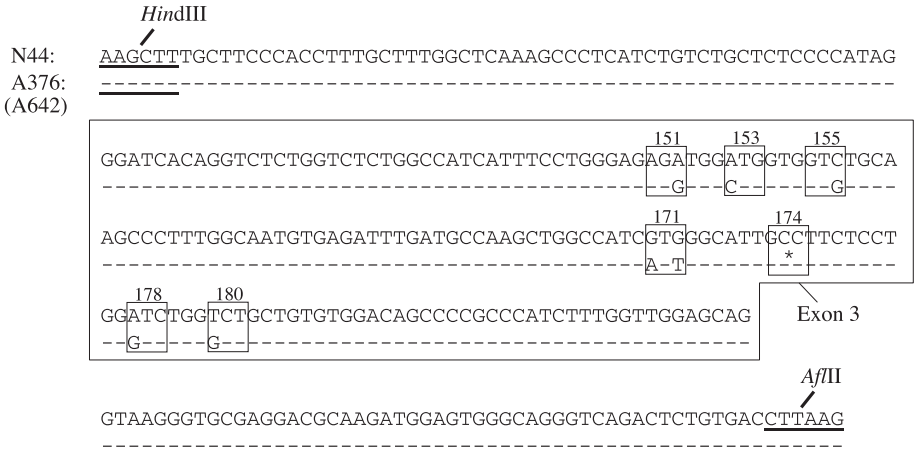

Fig. 3. Comparison of nucleotide sequence between N44 (L) and A376 (L) minigenes. In the genomic region between the *Hin*dIII and *Afl*II, no nucleotide-differences existed between the A376 (L) and A642 (L) minigenes, but seven nucleotide-differences existed between the N44 (L) and A376 (L) minigenes. Numbers above the boxes represent codon numbers. Codon 174, not different between these minigenes, is also highlighted by a box and an asterisk, as a polymorphism (C or T) is known there.

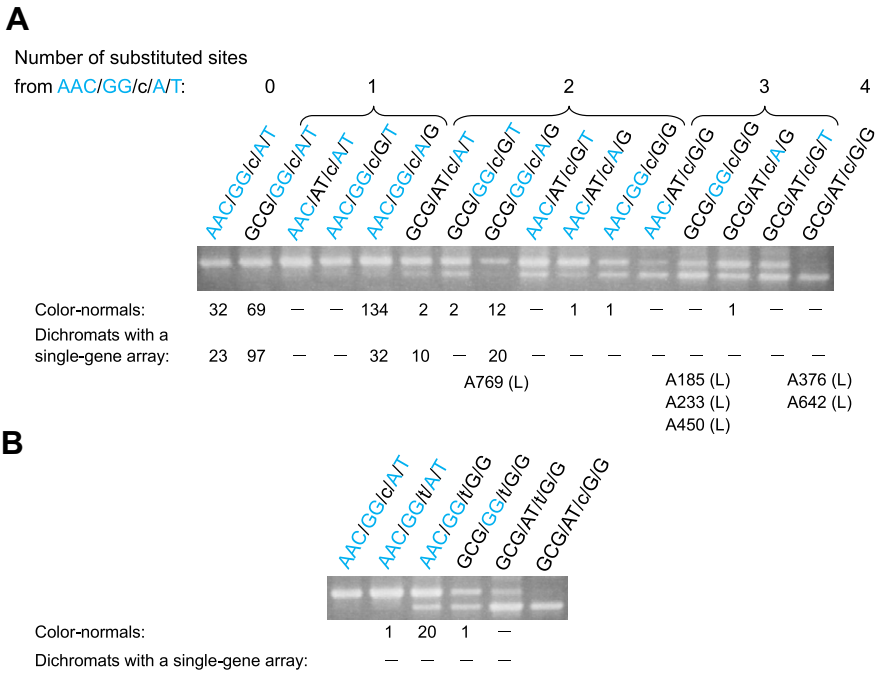

Fig. 4. RT-PCR analysis of the product from *in vitro* mutagenized minigenes. (A) Comparison of the RT-PCR products among the 16 possible haplotypes. The nucleotides of the N44 (L)-minigene which differed from the A376 (L)-minigene are shown in blue. The six participants having apparently normal L genes listed in Table 1 are shown below their corresponding haplotype. The number of normal L/M genes having each exon 3 haplotype is shown below the corresponding haplotype. The total number of the L/M genes was 276 in color-normals and 182 in dichromats, including those in (B). (B) Influence of the sixth nucleotide on the splicing pattern. The first and sixth lanes are controls, in which the sixth nucleotide is 'c'. The other lanes are those having 't' as the sixth nucleotide. The number of normal L/M genes having each exon 3 haplotype, except control lanes, is shown below the corresponding haplotype.

tion [15]. Crognale et al. [22] reported a case of blue-cone monochromacy, in which neither of L- and M-cones was functioning. The affected man had two L genes, both of which had the LIAVA combination. Mizrabi-Meissonnier et al. [16] reported on seven families with X-linked cone dystrophy/cone-rod dystrophy. One of the families had the LIAVA combination in the single M gene, and another family had the combination in the L gene in an array of L–M. These data indicate that the LIAVA combination must have a deleterious effect on the expression of opsin, but these authors have ascribed it, without any experimental data, to the protein's instability caused by the rare combination of amino acid residues. Our data presented in this study suggest that the exon 3 haplotype of 'GCG/AT/c/G/G' itself is the cause of color vision defect in these subjects.

It is of interest to study what factors concern recognition of exon 3 and which sequence of exon 3 is needed for the recognition. We checked the two haplotypes of exon 3, that of A376 (L) and N44 (L), using the Human Splicing Finder software (http://www.umd.be/HSF). Splicing enhancer motifs were frequently detected in two regions: the region containing $A^{151\text{-}3}$, $A^{153\text{-}1}$ and $C^{155\text{-}3}$, and the region containing $A^{178\text{-}1}$. On the other hand, splicing silencer motifs were frequently detected in the same regions but containing $G^{151\text{-}3}$, $C^{153\text{-}1}$ and $G^{155\text{-}3}$, and $G^{178\text{-}1}$. Therefore, among the eight polymorphic sites constituting the haplotypes of exon 3, the first three (A, A and C, or G, C and G) and the seventh one (A or G) are thought to have great influence on the splicing pattern. Further study is needed to clarify the exact control mechanisms for the recognition of exon 3.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.06.094.

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