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Molecular Determinants of Human Red/Green Color Discrimination

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Summary

The human red and green color vision pigments are identical at all but 15 of their 364 amino acids, and yet their absorption maxima differ by 31 nm. In an extensive mutagenesis study, including a set of 28 chimeric proteins modeled after pigments in the color-deficient human population and an additional 30 single and multiple point mutants, the spectral difference between these 2 pigments is shown to be determined by 7 and only 7 amino acid residues. In going from the red pigment to the green pigment, the 7 residues are $Ser^{116} \rightarrow Tyr$, $Ser^{180} \rightarrow Ala$, $Ile^{230} \rightarrow Thr$, $Ala^{233} \rightarrow Ser$, $Tyr^{277} \rightarrow Phe$, $Thr^{285} \rightarrow Ala$, and $Tyr^{309} \rightarrow Phe$.

Introduction

Human color vision is mediated by 3 visual pigments located in cone photoreceptor cells of the retina: the blue, green, and red color vision pigments (Boynton, 1979). The absorption maxima of the isolated pigments are 410, 532, and 563 nm for the blue, green, and red pigments, respectively (Oprian et al., 1991; Merbs and Nathans, 1992a; see Absorption Spectroscopy section of Experimental Procedures). Because 3 visual pigments are used, humans and other old world primates are trichromatic; we require three primary colors to match all others.

Inherited color vision defects are known for blue, green, and red color vision (Pokorny et al., 1979). Whereas inherited defects in blue color vision are very rare, affecting about 1 in 100,000 individuals, defects in the red and green regions of the spectrum are common. They are X-linked and affect about 8% of the male Caucasian population. Individuals with red/green color vision defects are dichromats or anomalous trichromats. Dichromats lack either the red pigment sensitivity (protanopes) or green pigment sensitivity (deuteranopes) and use only two primary colors to match all others. Anomalous trichromats require three primaries in color-matching tests, but they do not accept the proportions that satisfy color normal subjects. They require either more red (protanomalous) or more green (deuteranomalous) than the normal subjects.

In 1986, Nathans et al. (1986a) cloned the genes for the 3 color vision pigments, thus opening the way for a molecular genetic analysis of inherited red/green color vision deficiencies. The red and green genes are located on the X-chromosome with the red gene upstream of one or more green pigment genes (Vollrath et al., 1988). The nucleotide sequences of the red and green genes are highly similar, showing 98% identity. Both genes consist of six exons; exons 1 and 6 are identical for both genes. In an analysis of genomic Southern blots, the genes for the red and green pigments from individuals with inherited red/green color vision deficiencies (dichromats and anomalous trichromats) were observed to have undergone rearrangements (Nathans et al., 1986b). The rearrangements were proposed to produce chimeric proteins containing segments from both the red and the green pigments. The fact that the genes are highly similar and that they are located on the X-chromosome as a tandem array suggests that the chimeric pigments are formed as a result of unequal homologous recombination events between the two genes (Nathans et al., 1986b).

According to this model, 2 or more of the amino acid residues that are different in the 2 proteins determine the spectral difference between the 2 pigments (532 nm for the green versus 563 nm for the red). If the entire set of residues responsible for the spectral difference is transferred from 1 pigment to the other in the formation of a chimera, then also is the absorption maximum of that pigment converted from one to the other. An individual with this rearrangement would have effectively lost one of the 2 pigments and would therefore behave in color-matching tests as a dichromat. If instead, only a subset of the amino acids was transferred, the chimeric pigment would have spectral properties not of the red or green pigments, but rather it would have a spectrum with a maximum intermediate between that of the red and green pigments. An individual with this rearrangement would still possess 3 different pigments and would, therefore, still require three primaries in color-matching tests. Recently, Merbs and Nathans (1992b) have shown that chimeric proteins produced by the exchange of exons between the red and green genes do indeed have spectral properties which are intermediate between those of the parent red and green pigments and that exons 2, 3, 4, and 5 make contributions to the spectral difference between the 2 proteins.

To identify the individual amino acid residues that are responsible for the spectral difference between the red and green pigments, we have carried out an extensive mutagenesis study which includes all 28 possible chimeric pigments that could result from a single recombination event between the red and green genes (14 with green sequences 5′ upstream of the red and 14 with red sequences 5′ upstream of the green). We show here that 7 amino acids are responsible for the difference between the 2 pigments and that if these 7 amino acids, and only these 7, are changed in the green pigment the absorption spectrum becomes

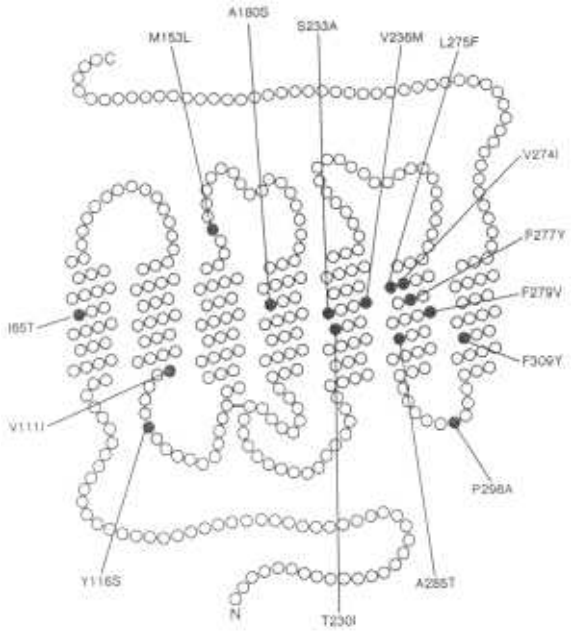

Figure 1. Schematic Diagram Showing the Structure of the Human Red and Green Color Vision Pigments

The red and green pigments are identical at all but 15 of the 364 amino acid residues in the proteins. The 15 amino acid differences are highlighted by solid circles in the figure. For each of the 15 amino acids, the identity of the amino acid residue in the green pigment is indicated, followed by the position in the amino acid sequence, followed by the identity of the amino acid residue in the red pigment. Position 180 is a site of polymorphism for the red pigment in the color normal population (Neitz and Jacobs, 1986; Neitz et al., 1989; Winderickx et al., 1992). About 40% of the population contains an Ala at this site and 60% a Ser. Absorption spectra for the 2 pigments corresponding to this polymorphism are shown in Figure 10A and have been reported by Merbs and Nathans (1992a).

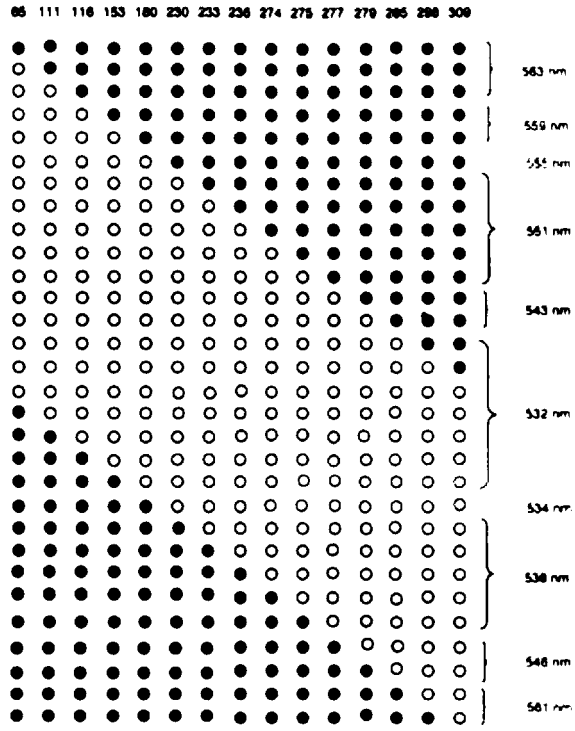

Figure 2. Schematic Representation of the Red/Green Chimeric Receptors

For each chimera, the identity of the 15 amino acids that differ between the red and green pigments (position identified at the top of the figure) is represented by a solid or open circle. A solid circle indicates that the amino acid is from the red pigment; an open circle indicates that the amino acid is from the green pigment (refer to Figure 1). Absorption maxima for the pigments are shown in the right-hand column. Maxima are shown only for pigments undergoing a spectral shift. The maxima are precise to within ±1 nm.

indistinguishable from the red pigment spectrum. These amino acids are (red → green): Ser^{116} → Tyr, Ser^{180} → Ala, Ile^{230} → Thr, Ala^{233} → Ser, Tyr^{277} → Phe, Thr^{285} → Ala, and Tyr^{309} → Phe.

Results

The human red and green pigments differ by 15 amino acids (Nathans et al., 1986a). The position number and corresponding amino acid identity at these 15 locations in the green and red pigments are indicated in Figure 1. To identify which of these amino acids are responsible for the spectral difference of the red and green pigments, we constructed 28 chimeric pigments (Figure 2). One set of chimeras was constructed by incrementally increasing the number of amino acids from the green pigment into the red pigment beginning at the amino-terminal end of the protein. Thus, the first chimera has amino acid 65 from the green pigment and the remaining amino acids from the red ($G^{65}R^{111-309}$), the second chimera contains amino acids 65 and 111 from the green and the remaining from the red ($G^{65-111}R^{116-309}$), and so on, as is shown schematically in Figure 2. The other set of chimeras was constructed similarly by incrementally increasing the number of red amino acids in the green pigment (Figure 2).

Absorption spectra for the purified and reconstituted chimeric pigments are shown in Figure 3 and Figure 4. The predominant spectral shifts in both sets of chimeras were observed for changes at positions 277 and 285. However, smaller shifts were observed from changes at other positions, although the changes were not necessarily identical in the two sets of mutants. For example, in the first set (amino-terminal green/carboxy-terminal red chimeras; Figure 3), spectral shifts were observed for changes at positions 116, 180, 230, 277, and 285, whereas in the second set (amino-terminal red/carboxy-terminal green chimeras; Figure 4), spectral shifts were observed for changes at positions 180, 230, 277, 285, and 309. Thus, there are 6 amino acid positions that significantly affect the spectral properties of the chimeric pigments: 116, 180, 230, 277, 285, and 309.

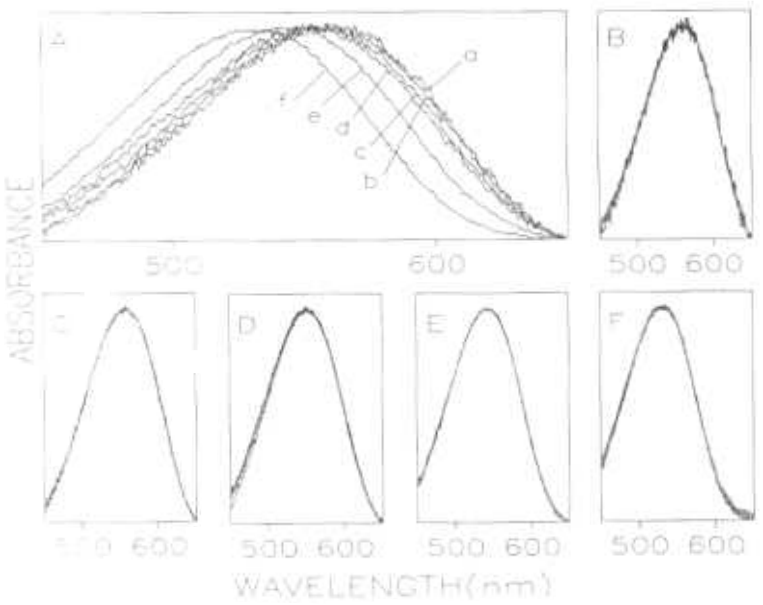

Figure 3. Absorption Spectra for the Set of Chimeras with Green Sequences 5' Upstream of the Red

(A) Spectra for all mutants showing a spectral shift: a, red wild type; b, $G^{65\text{-}116}R^{153\text{-}309}$; c, $G^{65\text{-}180}R^{230\text{-}309}$; d, $G^{65\text{-}230}R^{233\text{-}309}$; e, $G^{65\text{-}277}R^{279\text{-}309}$; and f, $G^{65\text{-}285}R^{290\text{-}309}$. Spectra for mutants not showing a spectral shift are shown in (B) $G^{65}R^{111\text{-}309}$, $G^{65\text{-}111}R^{116\text{-}309}$, and red wild-type; (C) $G^{65\text{-}116}R^{153\text{-}309}$ and $G^{65\text{-}153}R^{180\text{-}309}$; (D) $G^{65\text{-}230}R^{233\text{-}309}$, $G^{65\text{-}233}R^{236\text{-}309}$, $G^{65\text{-}236}R^{274\text{-}309}$, $G^{65\text{-}274}R^{275\text{-}309}$, and $G^{65\text{-}277}R^{277\text{-}309}$; (E) $G^{65\text{-}277}R^{279\text{-}309}$ and $G^{65\text{-}279}R^{285\text{-}309}$; and (F) $G^{65\text{-}285}R^{290\text{-}309}$, $G^{65\text{-}290}R^{309}$, and green wild-type. The spectrum for $G^{65\text{-}279}R^{277\text{-}309}$ in (D) shows a slight deviation from the other spectra in this figure along the short wavelength arm of the spectrum. This is found only in the difference spectrum and results from an unusual spectrum for the bleached pigment. The absolute spectra for the mutants in (D) are superimposable.

To determine the minimum subset of amino acids which could completely convert the spectrum of the green pigment into that of the red pigment, we constructed the set of mutants numbered 1-14 in Figure 5-Figure 7. Surprisingly, substitution of the 6 amino acids identified in the chimeric studies (116, 180, 230, 277, 285, and 309) was insufficient to shift the absorption spectrum from that of the green pigment to that of the red (mutant 13). The complete shift required in addition a change at position 233 (mutant 14; Figure 5 and Figure 7). Therefore, within our ability to resolve differences in the absorption maximum of these pigments, there are 7 and only 7 amino acid residues that are responsible for the entire spectral difference of the human red and green color vision pigments. These amino acids are (red → green): Ser^{116} → Tyr, Ser^{180} → Ala, Ile^{230} → Thr, Ala^{233} → Ser, Tyr^{277} → Phe, Thr^{285} → Ala, and Tyr^{309} → Phe.

The effect of single amino acid substitutions at positions 65, 180, 230, 233, 277, 285, and 309 in the red and green pigments was examined because the changes at these positions are nonhomologous; the side chain

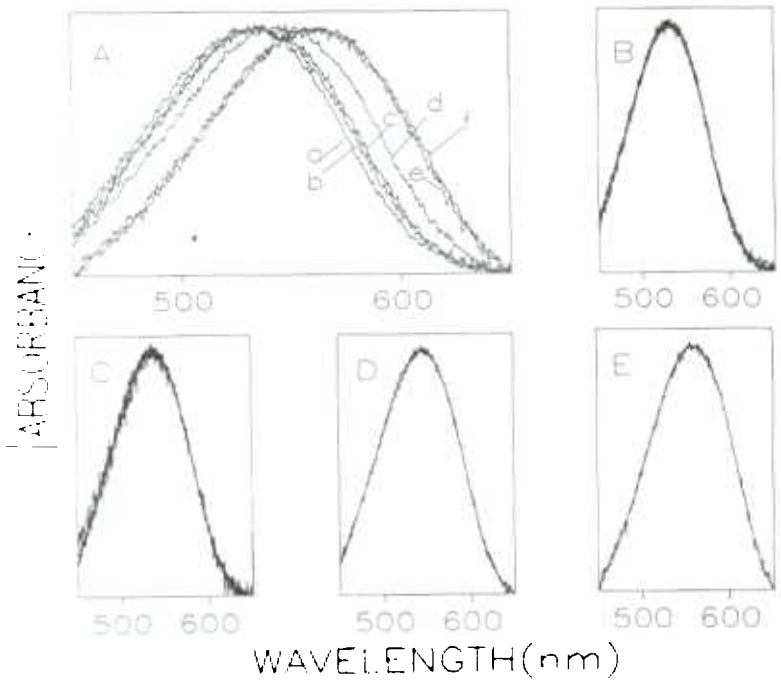

Figure 4. Absorption Spectra for the Set of Chimeras with Red Sequences 5' Upstream of the Green

(A) Spectra for all mutants showing a spectral shift: a, green wild-type; b, $R^{65\text{-}180}G^{230\text{-}309}$; c, $R^{65\text{-}230}G^{233\text{-}309}$; d, $R^{65\text{-}277}G^{279\text{-}309}$; e, $R^{65\text{-}285}G^{290\text{-}309}$; and f, red wild type. Spectra for mutants not showing a spectral shift are shown in (B) $R^{65}G^{111\text{-}309}$, $R^{65\text{-}111}G^{116\text{-}309}$, $R^{65\text{-}116}G^{153\text{-}309}$, $R^{65\text{-}153}G^{180\text{-}309}$, and green wild type; (C) $R^{65\text{-}230}G^{233\text{-}309}$, $R^{65\text{-}233}G^{236\text{-}309}$, $R^{65\text{-}236}G^{274\text{-}309}$, $R^{65\text{-}274}G^{275\text{-}309}$, and $R^{65\text{-}277}G^{277\text{-}309}$; (D) $R^{65\text{-}277}G^{279\text{-}309}$ and $R^{65\text{-}279}G^{285\text{-}309}$; and (E) $R^{65\text{-}285}G^{290\text{-}309}$ and $R^{65\text{-}290}G^{309}$.

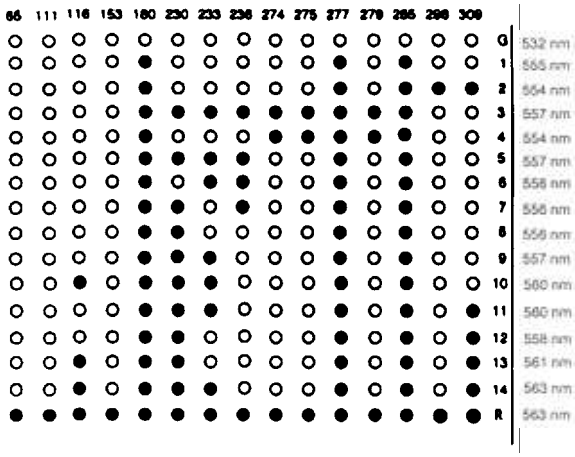

Figure 5. Schematic Representation of the Mutant Receptors Constructed to Determine the Minimum Number of Amino Acids Required to Convert the Spectrum of the Green Pigment Entirely to That of the Red Pigment (Mutants 1–14)

R and G refer to the wild-type red and green pigments, respectively. Absorption maxima for the pigments shown in the right-hand column are precise to within ±1 nm.

bears a hydroxyl group in 1 pigment, but not in the other. As is shown in Figure 8–Figure 10, spectral shifts were observed for substitutions at positions 180, 230, 233, 277, 285, and 309. In general, the spectral shifts were readily apparent in the red pigment and smaller or unresolved in the green. No change was observed upon substitution at position 65.

Discussion

From a comparative study of new world monkeys, Neitz et al. (1991) proposed that the spectral difference

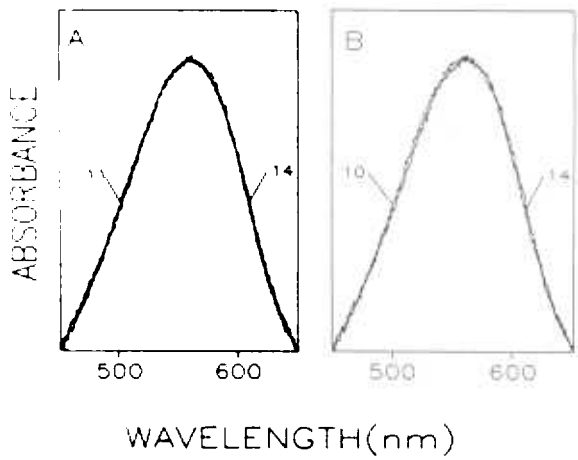

Figure 6. Influence of Amino Acids at Positions 116 and 309 in Shift of Green Pigment Spectrum to That of the Red Pigment

(A) Spectra for mutants 11 and 14. (B) Spectra for mutants 10 and 14. Schematic representation of mutants is in Figure 5.

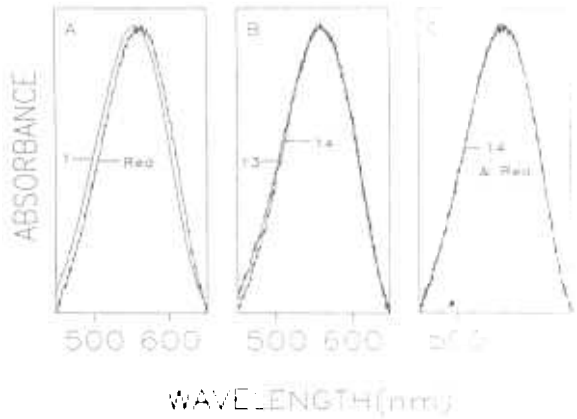

Figure 7. Amino Acids Required to Shift Green Pigment Spectrum to That of the Red Pigment

(A) Spectra for mutant 1 and red wild type. (B) Spectra for mutants 13 and 14. (C) Spectra for mutant 14 and red wild type. Schematic representation of mutants is in Figure 5.

between the human red and green color vision pigments could be attributed entirely to the amino acid residues at positions 180, 277, and 285. The involvement of these amino acids was also suggested by Yo-

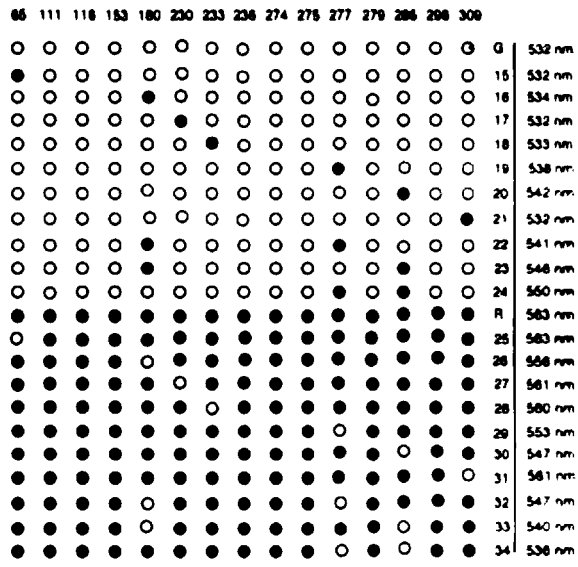

Figure 8. Schematic Representation of Single and Double Point Mutations at Positions for Which the Changes between Red and Green are Nonhomologous

Mutants 15 and 25 are $R^{65}G^{111-309}$ and $G^{65}R^{111-309}$, respectively, from Figure 2, Figure 3B, and Figure 4B. Absorption maxima for the pigments shown in the right-hand column are precise to within ±1 nm, with the following exceptions. First, maxima for mutants 27, 28, and 34 were each determined from only a single sample (spectra for mutants 27 and 28 are shown in Figure 9). The yield of mutant 34 was very low, and as a result our confidence in the accuracy of the absorption maximum is also low. In seven attempts, we were able to collect only a single spectrum for this mutant. Second, the range of maxima observed for mutant 33 was ±2 nm of the mean.

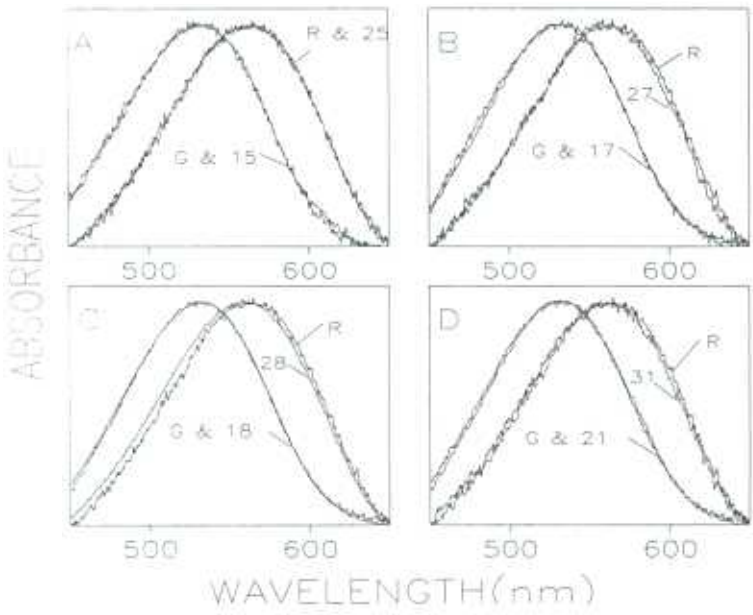

Figure 9. Absorption Spectra for Single Mutants at Positions 65, 230, 233, and 309

Each panel shows the effect of a single amino acid substitution in both the red and green pigments at positions 65 (A), 230 (B), 233 (C), and 309 (D). G, wild-type green; R, wild-type red. Schematic representations of the mutants are shown in Figure 8.

koyama and Yokoyama (1990) on the basis of a study of the blind cave fish Astyanax fasciatus and by Henderson and Schertler (1990) on the basis of a comparison of the structure of rhodopsin and the color vision pigments with that of bacteriorhodopsin (Henderson et al., 1990). Subsequent mutagenesis experiments by Chan et al. (1992) showed that these three positions, when substituted with hydroxyl-bearing amino acid side chains in the rod pigment rhodopsin, do indeed cause a red shift in the spectral absorption maximum of the pigment. These results are consistent with the data presented here in that a major portion of the spectral shift between the red and green color vision pigments is determined by the amino acids at positions 180, 277, and 285.

In addition, we have shown that amino acids at positions 116, 230, 233, and 309 are involved in the spectral shift. These conclusions are consistent with the studies of Winderickx et al. (1992) and Deeb et al. (1992) on inherited red/green color vision differences in humans, with a recent mutagenesis study (Merbs and Nathans, 1993), and with a study of photopigment polymorphism in the marmoset (Williams et al., 1992). Our results are also in agreement with a chimeric pig-

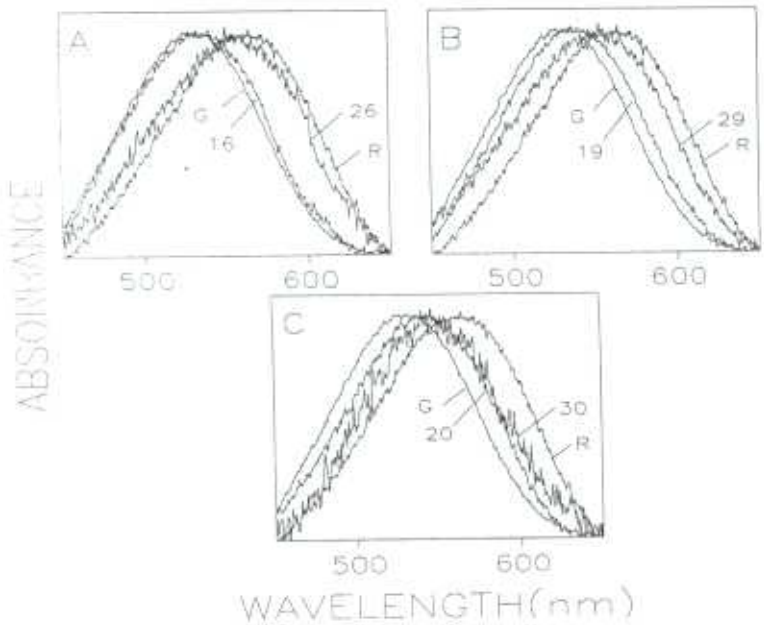

Figure 10. Absorption Spectra for Single Mutants at Positions 180, 277, and 285

Each panel shows the effect of a single amino acid substitution in both the red and green pigments at positions 180 (A), 277 (B), and 285 (C). G, wild-type green; R, wild-type red. Schematic representations of the mutants are shown in Figure 8.

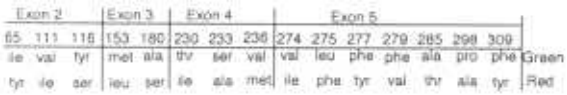

Figure 11. Schematic Diagram of the Red and Green Pigment Genes Showing Positions of the 15 Different Amino Acids within Exons 2-5

ment study from Merbs and Nathans (1992b) in which a spectral shift was observed for any chimera constructed with an exon containing at least 1 of the 15 amino acids that are different between the 2 proteins (i.e., exons 2, 3, 4, and 5; Figure 11). We attribute the shift from exon 2 to be a result of the amino acid at position 116, the shift from exon 3 to be due to 180, the shift from exon 4 to be due to 230 and 233, and the shift from exon 5 to be due to 277, 285, and 309.

Finally, we have shown that the absorption spectrum of the green pigment can be converted completely to that of the red pigment if and only if the amino acids at positions 116, 180, 230, 233, 277, 285, and 309 are changed to those found in the red pigment.

The mechanism by which these amino acid residues determine the spectral properties of the proteins is not known. They may affect the spectrum by a direct interaction with the chromophore or by an indirect effect on the structure of the chromophore-binding pocket. The effect of position 116 is most probably indirect since this residue is located outside of the membrane-embedded region of the protein in the extracellular loop between transmembrane segments II and III (i.e., it is unlikely to be in direct contact with the chromophore). As has been shown previously, Cl^- also induces a spectral shift in these pigments by an indirect mechanism since the Cl^--binding site is located in the extracellular loop between transmembrane segments IV and V (Wang et al., 1993). On the other hand, the correlation of hydroxyl-bearing amino acids with a red shift in the absorption spectrum is generally interpreted in terms of a direct electrostatic interaction of the polar side chains with the excited state of the chromophore (Mathies and Stryer, 1976; Chen et al., 1989). Even with Thr^{230} and Ser^{233}, the observation that removal of the hydroxyl-bearing side chains results in a spectral red shift (e.g., compare mutants 1, 8, and 9) can be explained by a specific interaction with the chromophore along the lines proposed in the "point-charge" model of Honig et al. (1979) if the hydroxyl groups interact with carbon centers in the retinal polyene chain which undergo an increase in charge density in the photoexcited state. Further studies will be required to elucidate the mechanistic details of these interactions.

Experimental Procedures

Materials

11-Cis-retinal was the generous gift of Peter Sorter and Hoffman-LaRoche (Nutley, NJ). CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate), egg yolk PC (L-α-phosphatidylcholine; type XI-E), HEPES, Sepharose 4B, dithiothreitol, and phenylmethylsulfonyl fluoride were from Sigma (St. Louis, MO). Peptide I (AspGluAlaSerThrThrValSerLysThrGluThrSerGlnValAlaProAla) was purchased from American Peptide Co., Inc. (Santa Clara, CA). The monoclonal antibody rhodopsin 1D4 (Molday and MacKenzie, 1983; MacKenzie et al., 1984) was purified from 20 l of hybridoma culture medium by $(NH_4)_2SO_4$ fraction and diethylaminoethyl-cellulose chromatography according to standard protocols, as has been described (Oprian et al., 1987). The rhodopsin 1D4 antibody was coupled to the Sepharose 4B solid support by the method of Cuatrecasas (1970).

Mutagenesis

The synthetic genes used in this work for the human red and green color vision pigments were designed to facilitate the mutagenesis procedures, and details concerning the construction and characterization of the genes can be found in earlier publications (Oprian et al., 1991; Wang et al., 1993). Mutagenesis was by the method of restriction fragment replacement (Lo et al., 1984), as has been described (Zhukovsky and Oprian, 1989; Zhukovsky et al., 1991, 1992; Robinson et al., 1992). All mutations were confirmed by DNA sequence analysis (Sanger et al., 1977).

Expression

Procedures for expression of the genes, reconstitution of the proteins with 11-cis-retinal, and purification of the pigments have been described (Oprian et al., 1991; Wang et al., 1993). Briefly, the genes were expressed transiently in COS cells, and the proteins were reconstituted with 11-cis-retinal before disruption of the cells with detergent. The pigments were solubilized from cell membranes in 0.75% CHAPS containing 0.8 mg/ml PC and then allowed to bind to an anti-rhodopsin 1D4-Sepharose 4B immunoaffinity matrix. After washing with the same detergent mixture, the pigments were eluted from the column with peptide I, and their spectral properties were determined. Purification of the red and green color vision pigments with the 1D4 anti-rhodopsin monoclonal antibody is made possible by the presence of an 8 amino acid epitope spliced onto the carboxyl terminus of the proteins (Oprian et al., 1991). Some of the chimeric pigments were unstable (specifically those from genes with 5'-red/green-3' sequences). In these cases, 30% (v/v) glycerol was added to the elution buffer, which improved the yield of pigment significantly with no apparent effect on the absorption spectrum.

Absorption Spectroscopy

Ultraviolet/visible absorption spectra were recorded on samples eluted from the anti-rhodopsin 1D4-Sepharose 4B immunoaffinity matrix using an Hitachi Model U-3210 spectrophotometer which was specifically modified by the manufacturer for use in a dark room. Data were acquired with the aid of an Everex System 1700 microcomputer using Spectra Calc software from Galactic Industries Corp. (Salem, NH). All spectra were recorded on samples of 1.0 cm path length in thermostated cell holders with the temperature maintained at 4°C. Unless noted otherwise, the data presented are difference spectra determined from samples before and after bleaching of the samples by exposure to light.

Multiple spectra were determined from at least two independent COS cell transfections for each mutant (except as noted in the legend to Figure 8). The maxima reported in Figure 2, Figure 5, and Figure 8 were determined from the first derivative of a fourth-order polynomial fit to a 40 nm region surrounding each maximum. The range of maxima observed for a given mutant did not exceed ± 1 nm of the mean. However, it is important to note that we did not rely on the absorption maxima to determine whether a spectral shift existed between two mutants. In general, if two mutants had very similar spectra, it was difficult to determine unambiguously whether a spectral shift existed between them solely on the basis of a determination of the positions of their absorption maxima. However, in all cases a shift was accompanied by a cross-over point between the two spectra.

At all wavelengths above the cross-over point, the spectrum for one mutant was higher in absorbance than the second, whereas at all wavelengths lower than the cross-over point, the absorbance of the second mutant was higher.

The yield of pigments as expressed by the maximal absorbance of a 400 μl sample isolated from ten plates of transfected COS cells ranged from 0.009 for mutant 30 (Figure 10) to 0.12 for mutant 1 (Figure 7). A sense of the yield of other pigments can be gained by visual comparison of the noise in the spectrum with that observed for mutants 1 and 30.

The absorption maxima quoted for the blue, green, and red pigments in the Introduction deserve further comment. The maxima for the green and red pigments, 532 and 563 nm, respectively, are from the work in this paper and were determined by difference spectroscopy. They differ slightly from the maxima reported in our previous study (530 and 560 nm for the green and red, respectively; Oprian et al., 1991), which were determined from absolute spectra. We attribute the difference to the effects of light scattering in the absolute spectra. Owing to spectral overlap with free retinal, the true maximum for the blue pigment cannot be determined directly from a difference spectrum. The 410 nm maximum quoted for the blue pigment is from absolute spectra of the blue pigment (Lee and Oprian, unpublished data) and is consistent with the 424 nm difference maximum reported in our previous study (Oprian et al., 1991). These maxima (410, 532, and 563) appear to be significantly different from those reported by Merbs and Nathans (426, 530, and 557; Merbs and Nathans, 1992a). We currently have no explanation for this difference.

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