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## Accelerated Publications

### Design, Chemical Synthesis, and Expression of Genes for the Three Human Color Vision Pigments<sup>†</sup>

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**ABSTRACT:** Color vision in humans is mediated by three pigments from retinal cone photoreceptor cells: blue, green, and red. We have designed and chemically synthesized genes for each of these three pigments. The genes were expressed in COS cells, reconstituted with 11-*cis*-retinal chromophore, and purified to homogeneity using an immunoaffinity procedure. To facilitate the immunoaffinity purification, each pigment was modified at the carboxy terminus to contain an additional eight amino acid epitope for a monoclonal antibody previously used to purify bovine rhodopsin. The spectra for the isolated pigments had maxima of 424, 530, and 560 nm, respectively, for the blue, green, and red pigments. These maxima are in excellent agreement with the maxima previously observed by microspectrophotometry of individual human cone cells. The spectra are the first to be obtained from isolated human color vision pigments. They confirm the original identification of the three color vision genes, which was based on genetic evidence [Nathans, J., Thomas, D., & Hogness, D. S. (1986) *Science* 232, 193].

**H**uman color vision is mediated by three visual pigments present in retinal cone photoreceptor cells [cf. Boynton (1979)]. The spectra for these pigments have been determined by microspectrophotometry of individual human cone cells (Dartnall et al., 1983). The spectra show absorption maxima at 420, 530, and 560 nm for the blue, green, and red cone pigments, respectively. Despite their very different absorption

maxima, these pigments all contain an identical 11-*cis*-retinal chromophore covalently attached to the protein by means of a Schiff base linkage to the  $\epsilon$ -amino group of a conserved lysine residue. Thus, the different absorption maxima for the pigments arise from differences in the amino acid sequence of the individual proteins and in the interaction of these amino acids with the chromophore.

Several years ago, Nathans et al. (1986a,b) cloned the genes for the blue, green, and red pigments using a homologous rhodopsin probe. The genes were identified by the following genetic criteria.

**Blue Gene.** Only one gene was localized to an autosome, chromosome 7. This gene was concluded to be that of the blue

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pigment since blue color vision deficiencies are known to be inherited autosomally.

**Green and Red Genes.** Two of the three genes were localized to the X chromosome. These were identified as green and red because inherited green/red color vision deficiencies are known to be X-linked. Discrimination between the green and red genes was based on genomic Southern blots from individuals with known color vision deficiencies.

The identification of the color vision genes rests largely on indirect evidence. A demonstration that these three genes code for proteins with the expected spectral properties has not hitherto been provided. We present here the first direct demonstration that the blue, green, and red genes do in fact code for proteins that, when reconstituted with 11-*cis*-retinal, have absorption maxima of 424, 530, and 560 nm, respectively.

#### EXPERIMENTAL PROCEDURES

**Materials.** 11-*cis*-Retinal was the generous gift of Peter Sorter and Hoffman-LaRoche (Nutley, NJ). CHAPS,<sup>1</sup> egg yolk PC (type XI-E), HEPES, Sepharose 4B, DTT, and PMSF were from Sigma (St. Louis, MO). Dodecyl  $\beta$ -D-maltoside was from Calbiochem (La Jolla, CA). Peptide I (Asp-Glu-Ala-Ser-Thr-Thr-Val-Ser-Lys-Thr-Glu-Thr-Ser-Gln-Val-Ala-Pro-Ala) was purchased from American Peptide Co., Inc. (Santa Clara, CA). The monoclonal antibody rho 1D4 (Molday & MacKenzie, 1983; MacKenzie et al., 1984) was purified from 20 L of hybridoma culture medium by (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> fractionation and DEAE-cellulose chromatography, according to standard protocols as have been described (Oprian et al., 1987). The rho 1D4 antibody was coupled to the Sepharose 4B solid support by the method of Cuatrecasas (1970).

**Design and Chemical Synthesis of the Human Color Vision Genes.** The principles that were used in the design of the genes were discussed in previous publications (Ferretti et al., 1986; Oprian et al., 1986). The overriding consideration was to introduce a large number of unique restriction sites into the gene sequence to facilitate later mutagenesis studies. This was accomplished without altering the amino acid sequence of the proteins by taking advantage of the degeneracy inherent in the genetic code.

The reagents and procedures used for chemical synthesis of the color vision genes were essentially as those used for synthesis of the genes for bovine rhodopsin (Ferretti et al., 1986; Oprian et al., 1986) and  $\beta_2$ -adrenergic receptor (McPhee and Oprian, manuscript in preparation).

**Expression of the Color Vision Genes.** The genes were expressed in COS cells following transfection with DEAE-dextran, virtually as has been described for expression of the bovine rhodopsin gene (Oprian et al., 1987; Franke et al., 1988; Zhukovsky & Oprian, 1989; Zhukovsky et al., 1991). Cells were harvested 72 h posttransfection for reconstitution and purification of the pigments. The level of expression is 2–3-fold less than that achieved with rhodopsin. This corresponds to about 1  $\mu$ g/100-mm culture dish (a 100-mm culture dish contains about  $1 \times 10^7$  cells and 2 mg of total protein).

**Reconstitution and Purification of the Pigments.** The following procedure was used for the green and red pigments. Unless specifically noted to the contrary, all procedures were performed at 4 °C or on ice. Typically, a single protein purification began with 10–20 culture dishes (100-mm diameter)

of transfected COS cells. The cells were washed twice while still attached to the plates with 5 mL/plate of buffer P, which contained 50 mM HEPES, pH 6.6, 140 mM NaCl, and 1 mM DTT. The cells were harvested by adding 1 mL/plate of buffer P and then scraping with a rubber policeman, followed by collection in a clinical centrifuge (3 min at approximately 1000g). The cell pellet was washed by resuspending the cells and then pelleting in the clinical centrifuge two additional times with 1 mL/plate of buffer P. The cells were then incubated for 30 min with 20  $\mu$ M 11-*cis*-retinal in buffer P (1 mL/plate). The incubation with retinal and all subsequent procedures were performed in the dark or under dim red illumination (15-W bulb with a Kodak No. 2 Safelight filter).

The proteins were solubilized from cell membranes essentially as has been described by Okano et al. (1989) for the resolution and purification of chicken visual pigments. Cells were collected by centrifugation, and the pellet was resuspended in 1 mL/plate of buffer S (buffer P containing 0.75% CHAPS, 0.8 mg/mL PC, 1 mM MnCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, and 1 mM PMSF). Membranes were allowed to solubilize for 1 h. The suspension was then spun in a clinical centrifuge to remove nuclei and incubated with rho 1D4–Sepharose 4B as has been described for rhodopsin (Oprian et al., 1987) except that buffer S was used throughout the purification. The pigments were eluted from the column with 370  $\mu$ L of 50  $\mu$ M peptide I in buffer S.

The procedure used for the blue pigment was essentially identical to that described for the green and red except that dodecyl maltoside was used instead of CHAPS/PC for solubilization and purification of the protein. The initial solubilization was in 1% dodecyl maltoside; subsequent washing and elution of the immunoaffinity matrix was in 0.1% dodecyl maltoside.

**Absorption Spectroscopy.** UV/visible absorption spectra were recorded on samples eluted from the rho 1D4–Sepharose 4B immunoaffinity matrix using an Hitachi Model U-3210 spectrophotometer that 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. Difference spectra were determined from samples before and after bleaching in the presence of 100 mM hydroxylamine (Wald, 1968; Wald et al., 1955).

#### RESULTS

**Design and Chemical Synthesis of the Genes.** Genes for each of the three color vision pigments were designed such that they coded for the naturally occurring amino acid sequence of the proteins inferred from cDNA clones (Nathans et al., 1986a). The genes for each pigment contained, in addition to the wild-type sequence, 8 amino acids at the carboxy terminus derived from the sequence of rhodopsin. These eight additional amino acids, Glu-Thr-Ser-Gln-Val-Ala-Pro-Ala, correspond exactly to the carboxy terminus of rhodopsin and are known to be the epitope for the monoclonal antibody rho 1D4 (MacKenzie et al., 1984) used previously for purification of rhodopsin from transfected COS cells. We knew from previous studies that this epitope could be placed on heterologous proteins and used for their purification with the rho 1D4 antibody (McPhee and Oprian, manuscript in preparation). Therefore, we placed the 8 amino acid epitope at the carboxy terminus of each pigment to allow their purification from COS cells. The spectra reported in this paper were obtained from pigments containing the rho 1D4 epitope.

<sup>1</sup> Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; PC, L- $\alpha$ -phosphatidylcholine; HEPES, N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid; DTT, DL-dithiothreitol; PMSF, phenylmethanesulfonyl fluoride.

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EcoRI      FapI      20              40              XhoI  HaeI      ApeI/Eco0109
AATTCACCC ATG CGC AAG ATG TCC GAG GAG GAG TTC TAC CTG TTC AAG AAC ATC TCG AGC GTC GGC CCT TGG GAC GGG CCC CAG
MET ARG LYS MET SER GLU GLU GLU PHE TYR LEU PHE LYS ASN ILE SER SER VAL GLY PRO TRP ASP GLY PRO GLN

100              120              AvaiI 140              160 BsaI
TAC CAC ATC GCT CCT GTC TGG GCT TTC TAC CTC CAG GCT GCT TTC ATG GGG ACC GTG TTC CTC ATC GGT TTC CCT CTG AAT
TYR HIS ILE ALA PRO VAL TRP ALA PHE TYR LEU GLN ALA ALA PHE MET GLY THR VAL PHE LEU ILE GLY PHE PRO LEU ASN

NcoI      BclI      200              DraI  220 FokI      240
GCC ATG GTG CTC GTG GCC ACC CTG CGT TAC AAG AAG CTG CGA CAG CCT TTA AAC TAC ATC CTG GTG AAC GTG TCC TTC GGT
ALA MET VAL LEU VAL ALA THR LEU ARG TYR LYS LYS LEU ARG GLN PRO LEU ASN TYR ILE LEU VAL ASN VAL SER PHE GLY

HinfI      260              280              NheI      300              AatII/AhaII
GGA TTC CTG CTG TGT ATC TTC TCC GTG TTC CCT GTG TTC GTG GCT AGC TGC AAC GGT TAC TTC GTG TTC GGA CGT CAC GTG
GLY PHE LEU LEU CYS ILE PHE SER VAL PHE PRO VAL PHE VAL ALA SER CYS ASN GLY TYR PHE VAL PHE GLY ARG HIS VAL

340      AvrII/KpnI      360      BstEII/DraII/NciI      SfiI/BclI      AsuII
TGT GCT CTG GAG GGT TTC CTA GGT ACC GTG GCT GGT CTG GTC ACC GGG TGG TCT CTG GCC TTC CTG GCC TTC GAA CGC TAC
CYS ALA LEU GLU GLY PHE LEU GLY THR VAL ALA GLY LEU VAL THR GLY TRP SER LEU ALA PHE LEU ALA PHE GLU ARG TYR

NdeI 420              440              NluI/FnuDII/HpaI/ScaI      480
ATC GTC ATA TGC AAG CCT TTC GGT AAC TTC AGG TTC TCC TCC AAG CAC GCG TTA ACC GTA GTA CTG GCT ACC TGG ACA ATC
ILE VAL ILE CYS LYS PRO PHE GLY ASN PHE ARG PHE SER SER LYS HIS ALA LEU THR VAL VAL LEU ALA THR TRP THR ILE

500ClaI      520              BspMI/XbaI/StuI      PvuII560
GGT ATC GGT GTA TCG ATC CCT CCC TTC TGT TGG TCT CGC TTC ATT CCG GAA GGC CTT CAG TGC AGC TGT GGC CCT GAC
GLY ILE GLY VAL SER ILE PRO PRO PHE PHE GLY TRP SER ARG PHE ILE PRO GLU GLY LEU GLN CYS SER CYS GLY PRO ASP

580      PvuII600      620              640      DdeI
TGG TAC ACC GTG GGT ACA AAG TAC CGA TCG GAG AGC TAC ACC TGG TTC CTG TTC ATC TTC TGC TTC ATC GTG CCT CTG AGC
TRP TYR THR VAL GLY THR LYS TYR ARG SER GLU SER TYR THR TRP PHE LEU PHE ILE PHE CYS PHE ILE VAL PRO LEU SER

660              680      SacI      700              HaeII
CTG ATC TGC TTC TCC TAC ACC CAA CTG CTG CGA GCT CTG AAG GCT GTG GCT GCT CAA CAG CAG GAG AGC GCT ACC ACC CAG
LEU ILE CYS PHE SER TYR THR GLN LEU LEU ARG ALA LEU LYS ALA VAL ALA ALA GLN GLN GLN GLU SER ALA THR THR GLN

740              XbaI      760              BsmBI      800      SnaBI
AAG GCT GAA AGG GAG GTG TCT AGA ATG GTG GTG GTG ATG GTG GGA TCC TTC TGC GTG TGC TAC GTA CCC TAC GCT GCT TTC
LYS ALA GLU ARG GLU VAL SER ARG MET VAL VAL VAL MET VAL GLY SER PHE CYS VAL CYS TYR VAL PRO TYR ALA ALA PHE

BstXI820      840              BglII      860              880              SphI
GCC ATG TAC ATG GTG AAC AAC AGG AAC CAC GGT TTA GAT CTG CGA CTG GTG ACA ATC CCT TCG TTC TTC TCC AAG TCC GCA
ALA MET TYR MET VAL ASN ASN ARG ASN HIS GLY LEU ASP LEU ARG LEU VAL THR ILE PRO SER PHE PHE SER LYS SER ALA

/NsiI      920              940HindIII/SfaNI      960
TGC ATT TAC AAC CCT ATC ATC TAC TGC TTC ATG AAC AAG CAG TTC CAA GCT TGC ATC ATG AAG ATG GTG TGC GGT AAG GCT
CYS ILE TYR ASN PRO ILE ILE TYR CYS PHE MET ASN LYS GLN PHE GLN ALA CYS ILE MET LYS MET VAL CYS GLY LYS ALA

980              BspMI/PstI      1000      AccI/SalI/Tth111I      1040
ATG ACC GAC GAG AGC GAC ACC TGC AGC TCC CAG AAG ACC GAG GTG TCG ACC GTG TCC TCC ACC CAG GTT GGC CCT AAC GAG
MET THR ASP GLU SER ASP THR CYS SER SER GLN LYS THR GLU VAL SER THR VAL SER SER THR GLN VAL GLY PRO ASN GLU

1060              1080 NotI
ACC TCC CAG GTG GCT CCT GCT TGA GC
THR SER GLN VAL ALA PRO ALA END

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FIGURE 1: Nucleotide and corresponding amino acid sequence for the synthetic blue gene. The DNA sequence shown is for the coding strand. The corresponding amino acid sequence is also indicated. Numbers refer to the nucleotide sequence beginning with the first A of the 5'-flanking *EcoRI* site. The gene was designed as an *EcoRI*-*NotI* restriction fragment containing 51 unique restriction sites within the coding sequence. The restriction sites are indicated by bold type above the sequence and by horizontal lines demarcating the recognition sequence. The gene contains the complete coding sequence for the wild-type pigment plus an additional eight codons at the carboxy terminus corresponding to the rho 1D4 epitope.

The sequences for the blue, green, and red genes are shown in Figures 1 and 2. The salient features of each are summarized as follows.

**Blue Gene.** The sequence for the blue gene is shown in Figure 1. It was constructed as an *EcoRI*-*NotI* restriction fragment. The gene is 1080 bp in length, including codons for the rho 1D4 epitope. The protein encoded by this gene is 356 amino acids in length. There are 51 unique restriction sites within the coding sequence that define restriction fragments for mutagenesis. The gene was assembled from a total of 38 oligonucleotides.

**Green and Red Genes.** The green and red pigments are highly homologous, differing by only 15 amino acids out of a total of 364. The sequences for the green and red genes are shown in Figure 2. They were constructed as *EcoRI*-*NotI* restriction fragments. The genes are 1130 bp in length, including codons for the rho 1D4 epitope. The proteins encoded by these genes are 372 amino acids in length. There are a total of 45 unique restriction sites in each sequence that define restriction fragments for mutagenesis. The green and red sequences are very similar and contain with one exception the same complement of unique restriction sites. The one position

different in the two genes is at about nucleotide 900, where the green gene has a *KpnI* site and the red gene has an *NsiI* site. Each gene was assembled from a total of 44 oligonucleotides.

The DNA sequence of all three genes was confirmed by sequence analysis of both strands using the dideoxy method (Sanger et al., 1977).

#### Absorption Spectra of the Isolated Color Vision Pigments.

The synthetic genes were inserted into a modified version of the expression vector pMT-2 (Franke et al., 1988) and then transfected into COS cells for production of the proteins. After reconstitution with 11-*cis*-retinal and purification using the rho 1D4 immunoaffinity matrix, the pigments were placed in a spectrophotometer for a determination of their absorption spectra. The spectrum recorded for the blue pigment is shown in Figure 3A, and the spectra for the green and red pigments are shown in Figure 3B. Due to interference from light scattering at shorter wavelengths, the absorption maximum for the blue pigment was determined from a difference spectrum (Figure 3A, inset). This was not necessary for the green and red pigments. As is shown, the maxima are 424, 530, and 560 nm for the blue, green, and red pigments, respectively.

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EcoRI   NcoI       20              40 HaeI              60   NdeI              80
AATTCACC ATG GCA CAG CAG TGG TCT CTC CAG AGG TTA GGC GGC AGG CAT CCT CAA GAC TCA TAT GAG GAC TCC ACC CAG
MET ALA GLN GLN TRP SER LEU GLN ARG LEU ALA GLY ARG HIS PRO GLN ASP SER TYR GLU ASP SER THR GLN

ClaI    100              MluI 120              AsuII 140              SacII 160
TCA TCG ATC TTC ACC TAC ACC AAC TCC AAC TCC ACG CGT GGC CCT TTC GAA GGC CCT AAC TAC CAC ATC GCT CCG CGG TGG
SER SER ILE PHE THR TYR THR ASN SER ASN SER THR ARG GLY PRO PHE GLU GLY PRO ASN TYR HIS ILE ALA PRO ARG TRP

AatII 180              200 65              220 DdeI              240
GTG TAC CAC CTG ACG TCC GTG TGG ATG ATC TTC GTG GTG ATC GCT TCC GTG TTC ACC AAC GGC TTA GTG CTG GCT GCT ACC
VAL TYR HIS LEU THR SER VAL TRP MET ILE PHE VAL VAL ILE ALA SER VAL PHE THR ASN GLY LEU VAL LEU ALA ALA THR
THR

DraI    260              280 HincII 300 BglII 320
ATG AAG TTT AAA AAG TTA CGC CAC CCT CTG AAC TGG ATT CTG GTC AAC CTG GCT GTG GCA GAT CTG GCT GAA ACC GTG ATC
MET LYS PHE LYS LYS LEU ARG HIS PRO LEU ASN TRP ILE LEU VAL ASN LEU ALA VAL ALA ASP LEU ALA GLU THR VAL ILE

EcoRV   111              116 360 AvrII 380              400
GCT TCC ACG ATA TCC GTG GTG AAC CAG GTG TAC GGT TAC TTC GTC CTA GGC CAC CCT ATG TGC GTG CTG GAG GGT TAC ACC
ALA SER THR ILE SER VAL VAL ASN GLN VAL TYR GLY TYR PHE VAL LEU GLY HIS PRO MET CYS VAL LEU GLU GLY TYR THR
ILE SER

HindIII 420              StuI 440              PvuII 460              153              480
GTA AGC TTA TGC GGT ATC ACA GGC CTG TGG TCT CTG GCT ATC ATC AGC TGG GAG CGC TGG ATG GTG GTG TGC AAG CCT TTC
VAL SER LEU CYS GLY ILE THR GLY LEU TRP SER LEU ALA ILE ILE SER TRP GLU ARG TRP MET VAL VAL CYS LYS PRO PHE
LEU

500 PvuI 520              540              180 PstI 560 NarI
GGT AAC GTG CGC TTC GAC GCT AAA CTG GCG ATC GTG GGT ATC GCT TTC TCC TGG ATC TGG GCT GCA GTG TGG ACG GCG CCT
GLY ASN VAL ARG PHE ASP ALA LYS LEU ALA ILE VAL GLY ILE ALA PHE SER TRP ILE TRP ALA ALA VAL TRP THR ALA PRO
SER

SspI    580 XbaI 600 AflII 640
CCA ATA TTC GGT TGG TCT AGA TAC TGG CCT CAC GGC CTT AAG ACC TCC TGC GGA CCG GAC GTG TTC TCC GGT TCC TCC TAT
PRO ILE PHE GLY TRP SER ARG TYR TRP PRO HIS GLY LEU LYS THR SER CYS GLY PRO ASP VAL PHE SER GLY SER SER TYR

SmaI    660 BstEII 680              230              233              236 720
CCC GGG GTG CAG TCC TAC ATG ATC GTG CTG ATG GTG ACC TGC TGC ATC ACC CCT TTA AGC ATC ATC GTG CTG TGC TAC CTC
PRO GLY VAL GLN SER TYR MET ILE VAL LEU MET VAL THR CYS CYS ILE THR PRO LEU SER ILE ILE VAL LEU CYS TYR LEU
ILE ALA MET

740 NheI 760 BalI 780 ScaI 800
CAG GTG TGG CTA GCT ATC CGC GCT GTG GGC AAG CAG CAG AAG GAG TCC GAG AGT ACT CAA AAG GCA GAG AAG GAA GTG ACC
GLN VAL TRP LEU ALA ILE ARG ALA VAL ALA LYS GLN GLN LYS GLU SER GLU SER THR GLN LYS ALA GLU LYS GLU VAL THR

BstXI 820 274 275 277 279 ApaI 860 285 SphI 880
AGG ATG GTG GTG GTG ATG GTG CTG GCT TTC TGC TTC TGC TGG GGC CCT TAC GCA TTC TTC GCA TGC TTC GCC GCT GCT AAC
ARG MET VAL VAL VAL MET VAL LEU ALA PHE CYS PHE CYS TRP GLY PRO TYR ALA PHE PHE ALA CYS PHE ALA ALA ALA ASN
ILE PHE TYR VAL THR

KpnI 900 298 (NsiI) 920 BglI/SfiI 309 940 960
CCT GGG TAC CCT TTC CAC CCT CTG ATG GCG GGC TTA CCG GGC TTC TTC GCT AAA TCC GCT ACC ATC TAC AAC CCT GTG ATC
PRO GLY TYR PRO PHE HIS PRO LEU MET ALA ALA LEU PRO ALA PHE PHE ALA LYS SER ALA THR ILE TYR ASN PRO VAL ILE
ALA TYR

SnaBI 980 BspMII 1000 AccI 1020 1040 BamHI ScaI/HgiAI
TAC GTA TTC ATG AAC CGC CAG TTC CGG AAC TGT ATA CTC CAG CTC TTC GGT AAG AAG GTG GAC GAC GGA TCC GAA TTG AGC
TYR VAL PHE MET ASN ARG GLN PHE ARG ASN CYS ILE LEU GLN LEU PHE GLY LYS LYS VAL ASP ASP GLY SER GLU LEU SER

1060 1080 XhoI/PaeR71 1100 SpeI 1120 1130 NotI
TCC GCT TCC AAG ACC GAG GTG TCC TCC GTC TCG AGC GTG TCT CCT GCC GAG ACT AGT CAG GTG GCT CCT GCT TAA GC
SER ALA SER LYS THR GLU VAL SER SER VAL SER SER VAL SER PRO ALA GLU THR SER GLN VAL ALA PRO ALA END

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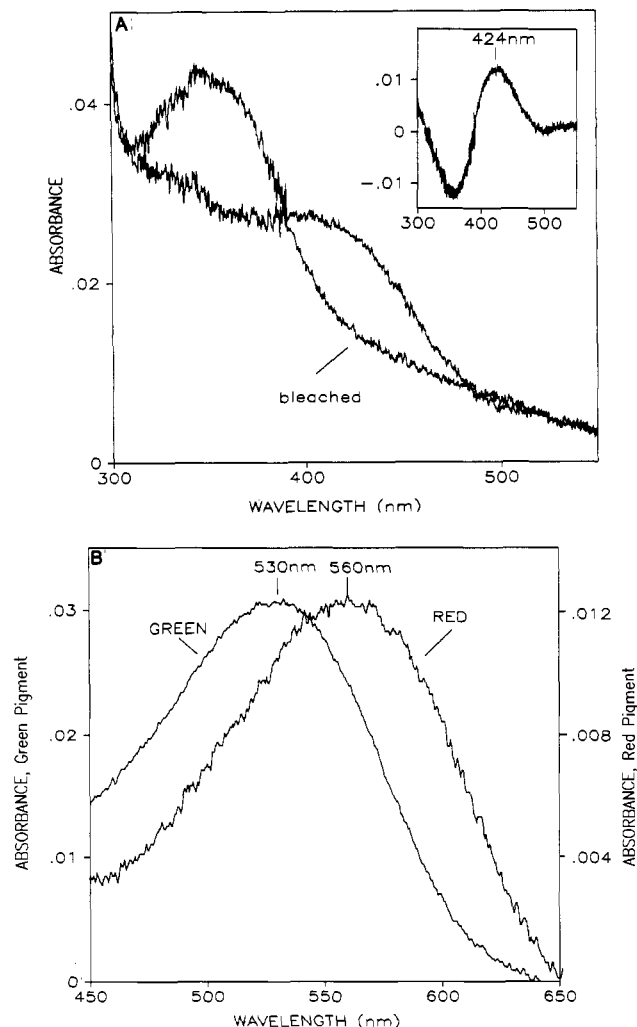
FIGURE 2: Nucleotide and corresponding amino acid sequence for the synthetic green and red genes. The DNA sequence shown is for the coding strand of the green gene. The corresponding amino acid sequence is also indicated. The number, codon, and amino acid for each of the 15 positions that are different between the two proteins are highlighted by bold type, with the amino acid of the red pigment indicated below that of the green pigment. The codons used in the red gene for the 15 different amino acids are as follows: Thr<sup>65</sup>, ACA; Ile<sup>111</sup>, ATC; Ser<sup>116</sup>, AGC; Leu<sup>133</sup>, CTG; Ser<sup>180</sup>, TCT; Ile<sup>230</sup>, ATC; Ala<sup>233</sup>, GCT; Met<sup>236</sup>, ATG; Ile<sup>274</sup>, ATC; Phe<sup>275</sup>, TTC; Tyr<sup>277</sup>, TAC; Val<sup>279</sup>, GTG; Thr<sup>285</sup>, ACC; Ala<sup>298</sup>, GCA; Tyr<sup>309</sup>, TAC. The numbers shown that are not in bold type refer to the nucleotide sequence beginning with the first A of the 5'-flanking *EcoRI* site. The genes were designed as *EcoRI*-*NotI* restriction fragments containing 45 unique restriction sites within the coding sequence. The restriction sites are as indicated. The *KpnI* site at position 900 in the green gene is not present in the red; instead, the red gene contains an *NsiI* site (shown in parentheses). The genes contain the complete coding sequence for the wild-type pigments plus an additional eight codons at the carboxy terminus corresponding to the rho 1D4 epitope.

These values are in very good agreement with the maxima expected on the basis of spectra determined by microspectrophotometry of individual human cone cells (Darnall et al., 1983). These are the first spectra reported for isolated human pigments and provide a direct demonstration that the genes previously identified for the blue, green, and red pigments do in fact code for the blue, green, and red pigments.

## DISCUSSION

Rhodopsin, the visual pigment from rod photoreceptor cells, is a stable protein that is easily isolated in abundance from bovine retina. Consequently, a great deal is known about the biochemical and spectral properties of rhodopsin (Wald, 1968;

Dratz & Hargrave, 1983). In contrast, much less is known about cone cell pigments. Iodopsin, the red pigment from chicken cone cells, was originally isolated in soluble form by Wald (Wald et al., 1955). This protein has a similar absorption maximum to the human red pigment and shares about 80% homology in amino acid sequence with the human protein (Kuwata et al., 1990; Tokunaga et al., 1990). Iodopsin has been resolved from the other chicken cone pigments (Fager & Fager, 1980; Yen & Fager, 1984) and purified to homogeneity (Okano et al., 1989). With the exception of iodopsin, very little is known of other cone cell pigments. The human cone cell pigments (color vision pigments) have never before been isolated. We have provided here the first spectra recorded



**FIGURE 3:** Absorption spectra for the blue, green, and red pigments. (A) Absorption spectrum for the blue pigment. The figure shows the spectrum obtained from a sample of the blue pigment that had been purified from 20 plates of transfected COS cells. Due to the short-wavelength maximum of this pigment, the spectrum is much more affected by light scattering than are those of the green and red pigments. For this reason, the absorption maximum of the blue pigment was determined from a difference spectrum. After the absorption spectrum for the isolated blue pigment was recorded, the sample was bleached in the presence of 100 mM hydroxylamine and the bleached spectrum was recorded. Inset: difference spectrum obtained by subtracting the bleached spectrum from the one obtained prior to bleaching. The absorption maximum is 424 nm. (B) Absorption spectra for the green and red pigments. These spectra were obtained from samples of the pigments that were purified from 20 plates of transfected COS cells. The absorption maximum of the green pigment is 530 nm, while that of the red pigment is 560 nm.

from isolated human color vision pigments. These spectra confirm the original identification of the genes by Nathans et al. (1986a,b). The absorption maxima are in excellent agreement with those determined by Dartnall et al. (1983) using microspectrophotometry of individual human cone cells. This was reassuring since it was certainly conceivable that the extra eight amino acids of the rho 1D4 epitope on the carboxy terminus of the pigments may have affected their spectral properties.

The use of this epitope for purification of heterologous proteins may be generally applicable. In addition to the color pigments, we have used it to purify a synthetic  $\beta_2$ -adrenergic receptor from transfected COS cells (McPhee and Oprian, manuscript in preparation). The modified receptor has ligand binding properties that are indistinguishable from the wild type, and it activates adenylate cyclase in transfected CHO

cell lines as would be expected of the wild-type protein. We note, however, that the purification is not as efficient as it is for rhodopsin. Typically, we lose about 50% of the protein because of inefficient binding to the immunoaffinity column (not shown), whereas with rhodopsin we recover almost 100% of the expressed protein (Oprian et al., 1987). Presumably, this reflects a slightly lower affinity of the antibody for the epitope when it is placed in a foreign context. A longer sequence of amino acids from the carboxy terminus of rhodopsin does not improve the results. We have tried adding 12 amino acids, instead of 8, to the green and red proteins, but we found no difference in yield of purified protein (not shown). The longer epitope was also without effect on the position of absorption maxima. The loss resulting from the lower binding affinity might be alleviated by preparing a more concentrated extract from the COS cells.

Our future efforts will be directed toward understanding the mechanism of wavelength regulation in the color vision pigments. The three pigments contain an identical 11-*cis*-retinal chromophore, and yet they absorb light with very different maxima. The different spectral properties are brought about by specific interactions of the chromophore with the proteins (Neitz et al., 1991). Site-directed mutagenesis is a promising approach to the analysis of these interactions and their relative roles in wavelength modulation. Previous mutagenesis studies using rhodopsin as a model for the color vision pigments have met with little success in terms of identifying the amino acids in cone pigments that are involved in wavelength modulation (Zhukovsky & Oprian, 1989; Nathans, 1990). The availability of the three synthetic genes and the ability to express them in functional form will greatly facilitate the progress of this work. This experimental system will also greatly facilitate studies of red/green color vision deficiencies, which are thought to arise from hybrid pigments resulting from unequal homologous recombination of the red and green genes (Nathans et al., 1986b; Piantanida, 1988; Neitz et al., 1989).

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## Photolysis Intermediates of Human Rhodopsin<sup>†</sup>

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**ABSTRACT:** Photochemical studies were conducted on human rhodopsin at 20 °C to characterize the intermediates which precede the formation of metarhodopsin II, the trigger for the enzyme cascade mechanism of visual transduction. Human rhodopsin was prepared from eyes which had previously been used for corneal donations. Time resolved absorption spectra collected from 10<sup>-8</sup> to 10<sup>-6</sup> s after photolysis of human rhodopsin in detergent suspensions displayed biexponential decay kinetics. The apparent lifetimes obtained from the data are 65 ± 20 and 292 ± 25 ns, almost a factor of 2 slower than the corresponding rates in bovine rhodopsin. The spectra can be fit well using a model in which human bathorhodopsin decays toward equilibrium with a blue-shifted intermediate (BSI) which then decays to lumirhodopsin. Spectra and kinetic rate constants were determined for all these intermediates using a global analysis which showed that the spectra of the human intermediates are remarkably similar to bovine intermediates. Microscopic rate constants derived from this model are 7.4 × 10<sup>6</sup> s<sup>-1</sup> for bathorhodopsin decay and 7.5 × 10<sup>6</sup> s<sup>-1</sup> and 4.6 × 10<sup>6</sup> s<sup>-1</sup> for the forward and reverse reactions of BSI, respectively. Decay of lumirhodopsin to later intermediates was studied from 10<sup>-6</sup> to 10<sup>-1</sup> s after photolysis of rhodopsin in human disk membrane suspensions. The human metarhodopsin I ⇌ metarhodopsin II equilibrium appears to be more forward shifted than in comparable bovine studies.

**R**hodopsin, the sensory pigment of scotopic vision, is the most successfully characterized receptor protein, particularly in its bovine form. Extending this level of understanding to human rhodopsin is important because mutations affecting the rhodopsin amino acid sequence have recently been shown to cause human disease. It thus becomes vital to characterize human rhodopsin so that the defects in health-related variants can be understood mechanistically. It is also interesting to compare rhodopsins with different sequences because this photoreceptor protein has emerged as a model for other more elusive membrane receptors, strongly posing the question of how amino acid sequence affects membrane protein function. While bovine rhodopsin has been extensively characterized,

previous studies of human rhodopsin have been confined to the slower processes occurring after the signal initiated by light absorption has been transmitted to other proteins in the rod outer segment (ROS).<sup>1</sup> Here we characterize the more rapid stages which lead to the activated form of human rhodopsin which triggers the enzyme cascade in the human eye.

Dramatic spectral changes which follow exposure of rhodopsin to light attracted attention as early as the 19th century (Kuhne, 1878). Modern time-resolved spectral studies have expanded our knowledge of these changes and have shown that photolysis leads to a number of discrete intermediate states of the protein (Ottolenghi & Sheves, 1989; Birge, 1990). The importance of understanding the mechanism of rhodopsin function has gained even wider significance in recent years, first because at least one form of retinitis pigmentosa has been shown to be caused by a variety of point defects in the rhodopsin gene (Dryja et al., 1990, 1991; Heckenlively et al., 1991; Ingelhearn et al., 1991; Sung et al., 1991) and second because

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<sup>1</sup> Abbreviations: BSI, blue-shifted intermediate; EDTA, ethylenediaminetetraacetic acid; ROS, rod outer segments; TBS, Tris-buffered saline.