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Functional expression of human cone pigments using recombinant baculovirus: compatibility with histidine tagging and evidence for *N*-glycosylation

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Abstract Mammalian color vision is mediated by light-sensitive pigments in retinal cone cells. Biochemical studies on native mammalian cone visual pigments are seriously hampered by their low levels and instability. We describe a novel approach for their functional expression, employing the baculovirus system in combination with histidine tagging to allow future purification and structural analysis. The human red and green cone pigments are produced in relatively large amounts and can be detected by immunocytochemistry as well as by immunoblotting. Histidine tagging has no significant effect on the absorbance maxima. The first evidence is presented that these pigments are *N*-glycosylated.

Key words: Baculovirus; Functional expression; Human cone pigment; Histidine tag; Glycosylation; Spectroscopy

1. Introduction

Vertebrate color vision is mediated by cone photoreceptor cells in the retina, which express specific photosensitive membrane proteins (visual pigments) for this purpose. In order to study the molecular properties of these pigments two basic strategies can be followed. A straightforward method is the isolation of cone pigments from the retina of organisms of interest. This method can only reasonably be applied to retinas where cone cells are abundant, for example chicken [1,2] and lizard (*Gecko gecko*) [3–5]. In most higher vertebrates, however, the rod cells by far outnumber the cones and isolation of native cone pigments from these species has not been reported. Furthermore, vertebrate retinas usually contain more than one type of cone pigment. The great similarity between these pigments makes multiple purification steps necessary, which is further hampered by the relatively low stability of these membrane proteins in detergent solution [1]. As a second approach, functional expression of cone pigments in eukaryotic cells can be used. Here, a high degree of homology between pigments of the same species is not a problem, since only one pigment is expressed. Another advantage of functional expression is that mutants of the protein of interest can be synthesized for the analysis of structure-function relationships. On the other hand, the usually low expression levels of recombinant membrane proteins in eukaryotic expression sys-

tems are a serious disadvantage. Prokaryotic expression systems, which usually produce more abundant levels of heterologous proteins, so far have not offered an alternative, probably because of the complex nature of visual pigments requiring correct membrane translocation and folding and several post-translational modifications.

Recombinant human cone pigments have been produced in mammalian cell lines [6,7] and display normal absorbance spectra. Other functional properties could so far not be determined, however. In this paper the potential of the baculovirus expression system for the production of recombinant cone pigments is demonstrated. This expression system has been successfully employed for the production of wild type and mutant forms of the rod pigment bovine rhodopsin [8–11], and a highly efficient purification procedure was developed using histidine tagging in combination with immobilized metal affinity chromatography [12]. Hence, we adopted this approach and evaluated the baculovirus system for functional expression of histidine-tagged human red and green cone pigments (HRH and HGH, respectively). Both proteins could be functionally expressed at reasonable levels ($2\text{--}4 \times 10^6$ copies/cell). Furthermore, we present the first evidence for *N*-glycosylation and for targeting of the recombinant cone opsins to the plasma membrane. Upon regeneration with the chromophore 11-*cis* retinal normal spectral properties are observed. The successful expression of histidine-tagged pigments in suitable amounts presents excellent perspectives for rapid purification and eventual detailed functional and structural analysis in a native-like lipid environment.

2. Materials and methods

2.1. Construction of a 6×histidine tag at the 3' end of human red and green cone pigment cDNA

Synthetic cDNAs encoding the human red and green sensitive cone pigments were generously provided by Dr. D.D. Oprian (Brandeis University, Waltham, MA, USA) as an *EcoRI*-*NotI* construct in pSP65 [6]. Both cDNAs contain 8 extra codons at the 3' end coding for the 8 C-terminal amino acids in rhodopsin, which constitute the epitope for the monoclonal antibody 1D4. Instead, a C-terminal 6×histidine tag sequence was constructed by digestion of the cDNAs in pSP65 with *XhoI* and *NotI* and insertion of a ds DNA fragment with *XhoI* and *NotI* overhangs. This fragment consisted of two complementary oligonucleotides (overhangs underlined) 5'-tcgagcgtgagtcctgccaccatcaccatcaccattaagaattcgc-3' and 5'-ggccgcgaattcttaatgtgatggtgatggtggcaggactcaccg-3'. Insertion of this fragment led to the generation of an additional *EcoRI* restriction site immediately downstream of the stop codon (Fig. 1). The histidine tag-containing cDNAs could therefore be excised by *EcoRI* digestion. After ligation into *EcoRI* digested pUC19 the correct sequence was confirmed by dideoxy sequencing. The cDNAs were then ligated into *EcoRI* digested pVL1393 (PharMingen) resulting in the transfer plasmids pVL1393HRH (human red histidine tag) and pVL1393HGH (human

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Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; dpi, days post infection; HGH, histidine-tagged human green cone pigment; HRH, histidine-tagged human red cone pigment; RT, room temperature; v-RhoHis_{6x}, histidine-tagged bovine rhodopsin

green histidine tag). Transcription of both cDNAs is under the control of the polyhedrin promoter.

2.2. Generation and purification of recombinant baculovirus

The transfer plasmids pVL1393HRH and pVL1393HGH (2 µg) were each cotransfected with 200 ng BaculoGold DNA (PharMingen) according to the manufacturer's instructions. Recombinant baculoviruses were isolated and purified by several subsequent plaque assays [13].

2.3. Cell culture and viral infections

The *Spodoptera frugiperda* cell line IPLB-Sf9 was used to propagate the baculovirus. The cells were maintained at 27°C in TNM-FH medium supplemented with 10% (v/v) fetal calf serum (Life Technologies), 50 µg/ml streptomycin and 50 U/ml penicillin (Life Technologies). Cells were grown as monolayers in culture flasks (Greiner) or as 100 ml suspension cultures in 250 ml spinner flasks (Bellco). In suspension cultures Pluronic F-68 (Sigma) was added to a final concentration of 0.1% (w/v) during cell growth and 0.3% (w/v) during viral infection. Viral infection of monolayer cultures and suspension cultures was performed with a multiplicity of infection of 5. The cells were harvested 3 days post infection (dpi) by centrifugation (5 min, 1000×g, room temperature).

2.4. SDS-PAGE and immunoblotting

After harvesting, the insect cells were frozen and thawed twice in PBS (10⁶ cells/ml), followed by DNase (Sigma) treatment (1 µg/ml suspension) for 15 min at room temperature (RT). Proteins were solubilized [14] and separated by electrophoresis on a 12% SDS-polyacrylamide gel (BioRad, Mini Protean II). After separation the proteins were transferred to nitrocellulose sheets (Schleicher and Schuell, BA 85, pore size 0.45 µm) by electroblotting. The blots were probed overnight with antibody CERN956 (anti-red/green N-terminal, dilution 1:1000) or CERN9416 (anti-histidine tag, dilution 1:1000). Generation and characterization of these antibodies will be described elsewhere (DeGrip, Vissers and Bovee-Geurts, in preparation). After washing with PBS, the second antibody (swine anti-rabbit immunoglobulin, Dako Immunoglobulins, dilution 1:300) was added and incubated for 1 h at RT. The blots were then rinsed with PBS and incubated with the third antibody (rabbit peroxidase-anti-peroxidase, Dako Immunoglobulins, dilution 1:500) for 1 h followed by a rinse with PBS. For detection the substrate 4-chloro-1-naphthol (Merck; 0.3% (w/v) in PBS containing 16.5% (v/v) methanol and 0.02% (v/v) H₂O₂) was used. The blots were stained for maximally 10 min, washed in distilled water and dried.

2.5. Confocal laser scan microscopy

Sf9 cells were grown on coverslips to confluence and subsequently infected with the recombinant baculovirus (MOI=1–5). At 2 dpi the cells were washed twice with PBS and fixed in 1% paraformaldehyde in phosphate buffer pH 7.4 for 1 h at RT. The cells were permeabilized in methanol at –20°C for 5 min after which they were allowed to dry. Next, aspecific binding sites were blocked by incubation with blocking buffer (PBS, 0.05% (v/v) Tween-20 (Serva), 1% (w/v) gelatin, 2% (v/v) FCS) for 30 min, followed by washing with washing buffer (blocking buffer without gelatin or FCS). The cells were then incubated with the primary antibody (CERN956) in blocking buffer for 30–60 min. After washing twice the rhodamine-conjugated secondary

antibody (SAR-TRITC, Dako Immunoglobulins, 1:50 in blocking buffer) was added and incubated for 30 min at RT. The samples were then rinsed three times, followed by a short wash with distilled water. Next, the cells were dehydrated in methanol, dried and mounted in 10% Mowiol (Hoechst), 2.5% (w/v) NaN₃, 25% (v/v) glycerol in 0.1 M Tris-HCl, pH 8.5. Confocal laser scan microscopy was performed on a BioRad MRC 1000 confocal microscope. Images were averaged over 5 scans.

2.6. Regeneration of cone pigments

Recombinant baculovirus infected insect cells were harvested and resuspended in 3 times diluted buffer A (50×10⁶ cells/ml; buffer A: 20 mM PIPES, 140 mM NaCl, 20% (v/v) glycerol, 5 mM β-mercaptoethanol, 4 µM leupeptin, pH 6.5). The suspension was then homogenized by five strokes in a Potter-Elvehjem tube. After centrifugation (10 min, 20 000×g, RT) the pellet was resuspended in buffer A (80×10⁶ cells/ml) and β-dodecylmaltoside [15] was added to a final concentration of 0.5 mM. All subsequent manipulations were performed in the dark or under dim red light (Schot-Jena RG 650 cut-off filter). The visual pigments were regenerated by incubation with 11-*cis* retinal. An aliquot of 20 nmol, dissolved in hexane, was blown to dryness with argon and dissolved in 10 µl dimethylformamide and added to 100×10⁶ cells. The mixture was then incubated under argon for at least 1 h (RT). The pigments were solubilized by the addition of CHAPS (Sigma) to a final concentration of 1% (w/v). After 1 h (RT) the suspension was centrifuged (30 min 100 000×g, 4°C) and the supernatant was used for spectroscopy or glycosylation analysis.

2.7. Analysis of N-glycosylation

Regenerated and detergent-solubilized HGH and histidine-tagged bovine rhodopsin (v-RhoHis₆) [12] (0.8 nmol each) were incubated overnight at 37°C with 1 unit N-glycosidase F (Boehringer Mannheim) in 0.5 ml containing 0.1% (w/v) SDS, 0.6% (w/v) β-dodecylmaltoside and 2 µM leupeptin. Samples were prepared for SDS-PAGE [14] and 4 pmol of pigment was applied to the gel. After electroblotting, the blot was probed with antibody CERN9416 (1:500).

2.8. UV-Vis spectroscopy

Spectra were recorded on a Perkin Elmer Lambda 15 spectrophotometer and analyzed using SpectraCalc software (Galactic Industries Corp.). The samples were kept at a temperature of 10°C using a thermostatted cell holder. Hydroxylamine was added to a final concentration of 20 mM and spectra were recorded before and after illumination for 3 min with a 75 W light bulb equipped with a 450 nm cut-off filter. Difference spectra were obtained by subtracting the spectrum after illumination from that before illumination.

3. Results

3.1. Construction of the histidine-tagged cone pigment cDNAs

The introduction of a 6×histidine tag at the C-terminus of the human red and green pigments was achieved by insertion of a synthetic ds DNA fragment at the 3' end of the corresponding synthetic cDNAs. The histidine tags were constructed at the C-termini of the proteins (Fig. 1) because ex-

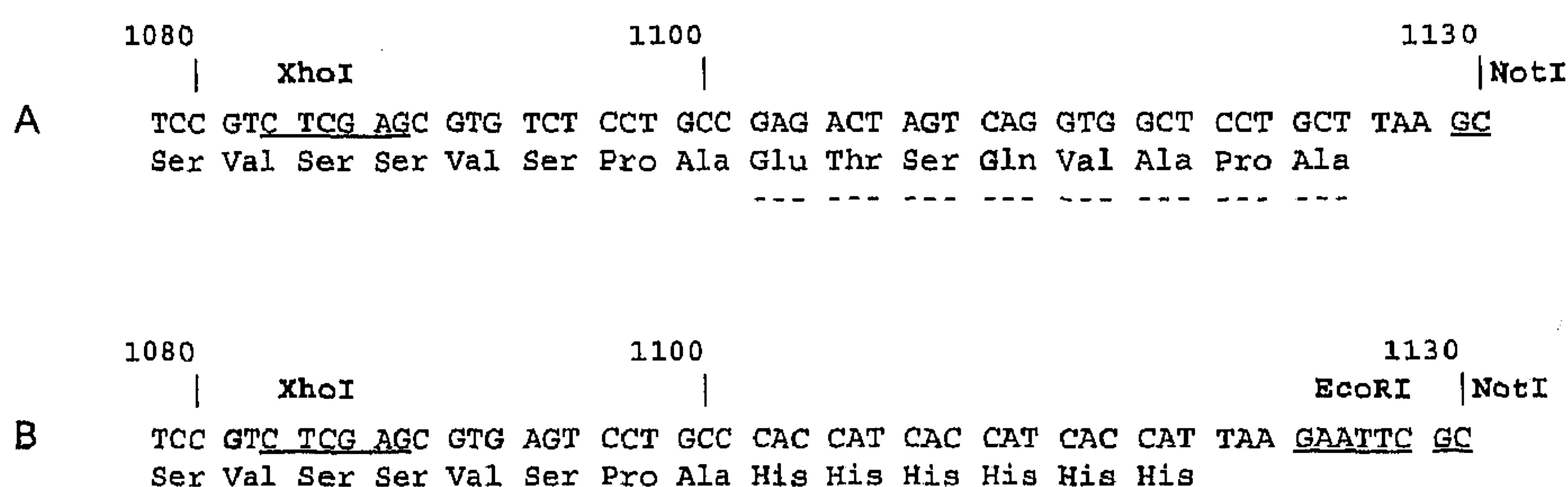


Fig. 1. Nucleotide sequences of the 3' end of the synthetic red and green cone pigment cDNAs. A: Sequence of the original cDNAs. The eight C-terminal codons (broken line) correspond to the rhodopsin 1D4 epitope [6]. B: Sequence of histidine-tagged red and green cone pigment cDNAs. Restriction sites are printed in bold face and are underlined in the DNA sequence.

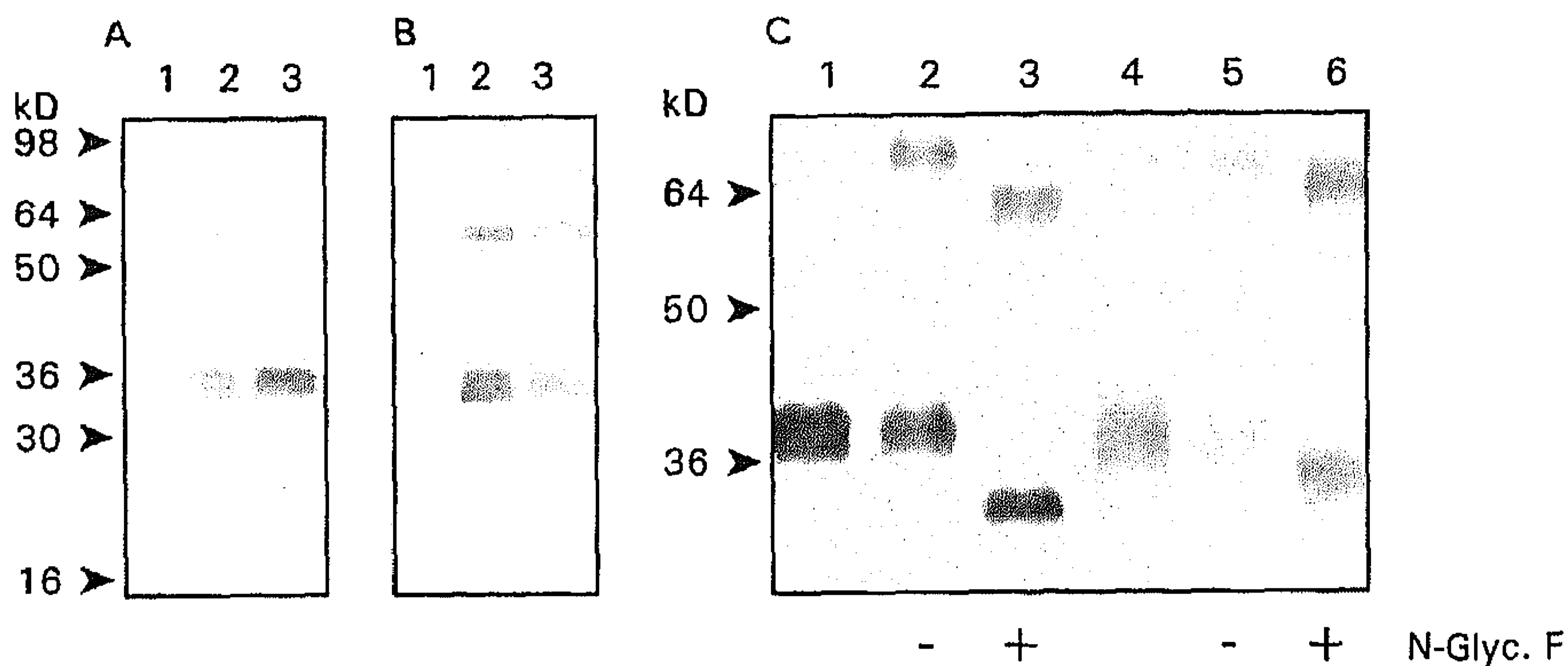


Fig. 2. A and B: Immunoblot of extracts of Sf9 cells infected with recombinant baculoviruses encoding HRH or HGH (50 000 cells/lane). The blot in A was probed with polyclonal antiserum CERN956 (1:1000), elicited against the N-terminal region of the red and green cone pigment. The blot in B was probed with polyclonal antiserum CERN9416 (1:1000), elicited against a histidine tag peptide. Lane 1: mock infected insect cells; lane 2: HRH infected insect cells; lane 3: HGH infected insect cells. Monomers migrate with an apparent molecular weight of approximately 35 kDa. Higher bands represent oligomers, which are artifacts of the SDS-solubilization. C: Effect of *N*-glycosidase F on the migration pattern of histidine-tagged bovine rhodopsin (*v*-RhoHis_{6x}) [12] and HGH. Regenerated and solubilized *v*-RhoHis_{6x} (lane 1) and HGH (lane 4) were incubated with (lanes 3 and 6) or without (lanes 2 and 5) *N*-glycosidase F.

tensions at this part of the pigment do not interfere with spectral properties studied thus far [6,16]. Both cDNAs were cloned in a transfer vector under transcriptional control of the baculovirus very late polyhedrin promoter, since relatively high expression levels were obtained with this promoter for the bovine rod pigment (up to 50 pmol/10⁶ cells) [11].

3.2. Expression of the cone pigment proteins in the insect cell line IPLB-Sf9 and evidence for *N*-glycosylation

Recombinant baculoviruses encoding the cone pigments were used to infect Sf9 cells. Protein extracts from the infected insect cells were separated by SDS-PAGE and probed on immunoblot using the polyclonal antisera CERN956 (elicited against an oligopeptide corresponding to an N-terminal region identical in the human red and green cone pigment, Fig. 2A) and CERN9416 (elicited against a histidine tag peptide, Fig. 2B). The immunostaining clearly demonstrates that the pigment proteins are expressed and migrate with an apparent molecular weight of approximately 35 kDa. The presence of the C-terminal histidine tag is confirmed by the reaction with CERN9416, and proves that full-length proteins are produced. The human red and green cone pigment sequence contain the same putative *N*-glycosylation site (Asn-34). In order to establish the glycosylation of the cone pigments, regenerated and solubilized HGH was incubated with *N*-glycosidase F, which specifically cleaves *N*-glycan chains from glycoproteins. Regenerated and solubilized histidine-tagged bovine rhodopsin (*v*-RhoHis_{6x}) was processed in a similar way and served as a reference. As is shown in Fig. 2C, incubation with *N*-glycosidase F results in a reduction in molecular weight for *v*-RhoHis_{6x} as well as for HGH. The larger shift for *v*-RhoHis_{6x} is explained by the fact that this pigment contains two putative glycosylation sites, compared to one for HGH. Comparison of lanes 1–2 and 4–5 demonstrates that overnight incubation promotes aggregation, but does not suffer from proteolytic breakdown and that the observed mobility shift is due to cleavage by *N*-glycosidase F.

3.3. Targeting of the pigment proteins

The sub-cellular localization of the histidine-tagged pigments was analyzed at 2 dpi by immunocytochemistry using

confocal laser scan microscopy (Fig. 3). At this stage HGH (Fig. 3A) is present in the cytosol, probably in membranous structures like endoplasmic reticulum, Golgi network or transport vesicles, and already has been partially targeted to the plasma membrane. For HRH (Fig. 3B) a similar distribution is apparent, although much less staining of the plasma membrane is observed.

3.4. Spectral properties

The cone pigment proteins were regenerated into photosensitive pigments by incubation of infected insect cells with 11-*cis* retinal and were subsequently solubilized by the addition of CHAPS. The solubilized samples were analyzed by UV-Vis spectroscopy. The λ_{\max} of the green pigment can be read directly from difference spectrum (Fig. 4A). In the difference spectrum of the red pigment, however, some spectral impurities are present (probably of heme pigment origin) that disturb the spectrum and hamper direct reading of the λ_{\max} . Accordingly, λ_{\max} values were calculated by fitting the long wavelength limb of the absorption curve to a template derived by Partridge and DeGrip [17], designed for vitamin A1-based visual pigments. In this way a λ_{\max} was obtained of 560 ± 1 nm and 527 ± 1 nm, for the red and green cone pigment, respectively. For comparison, we also expressed a non-histidine tag containing (wild type) human red cone pigment, which displayed a maximum sensitivity at 555 ± 1 nm (Fig. 4B). Besides the absence of the histidine tag, the latter pigment also differs in the amino acid present at position 180 (a polymorphic site in the red pigment [18]). The wild type pig-

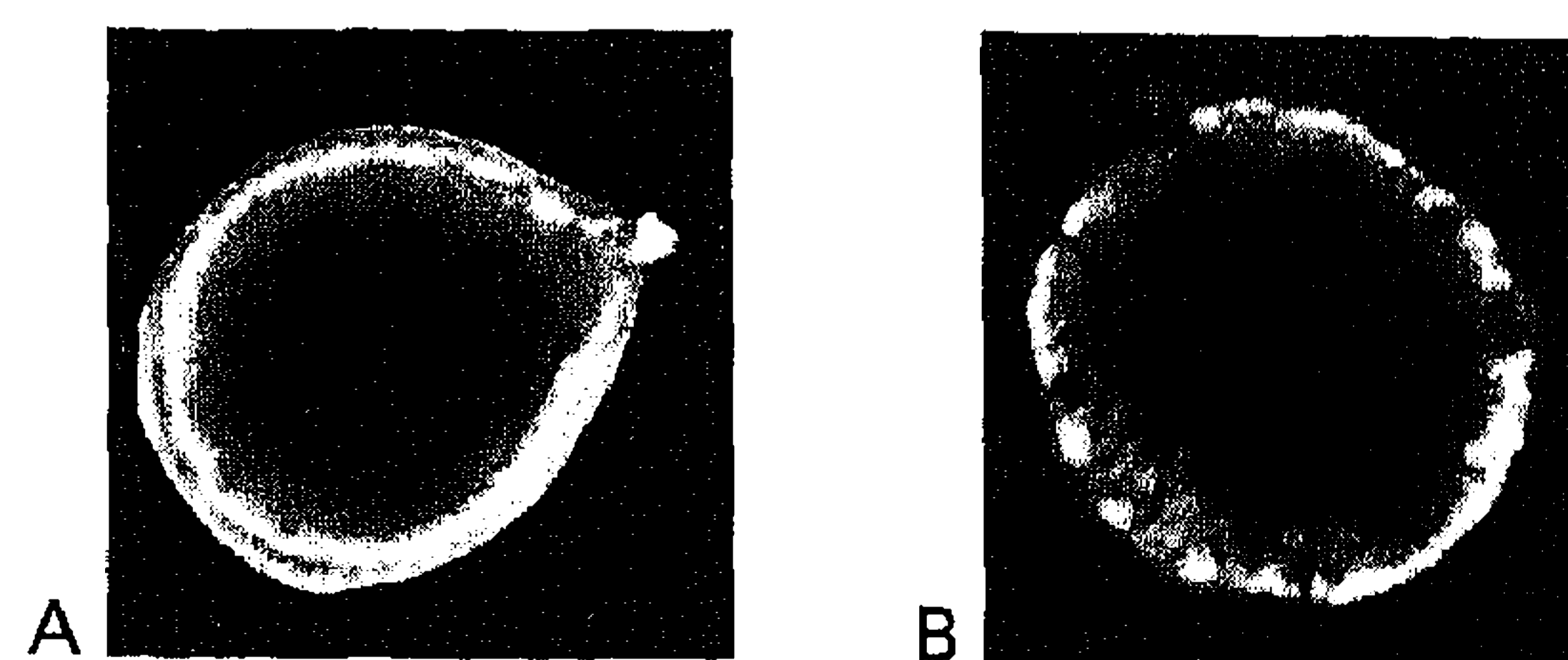


Fig. 3. Confocal images of insect cells expressing HGH (A) and HRH (B). The pigments were detected with antibody CERN956.

ment has alanine at this position, whereas the histidine-tagged pigment contains serine.

In all difference spectra of the cone pigments we obtained, as well as in those of native and recombinant bovine rhodopsin the ratio of the positive absorbance peak (pigment) to the negative peak representing the retinaloxime (produced upon illumination) is approximately 1:1. Therefore, the molar amount of the expressed histidine-tagged pigments was calculated assuming that the molar absorbance coefficients of the pigments are comparable to that of bovine rhodopsin (40 000 l/mol·cm). Yields were optimal at about 3 dpi and amounted to 2.0 ± 0.5 pmol/ 10^6 cells and 3.5 ± 1 pmol/ 10^6 cells for the red and green pigment, respectively. These expression levels correspond roughly to $2\text{--}4 \times 10^6$ copies of functional pigment per insect cell.

4. Discussion

Cone pigments constitute a group of proteins of which little is known with respect to their biochemical properties. This is in strong contrast to the rod pigment rhodopsin. The latter protein can be easily isolated in large quantities from bovine retinas and consequently its working mechanism is under detailed investigation. Because of their low abundance in mammalian retinas, functional expression of recombinant cone pigments offers a potential way to investigate these proteins in more detail. So far, functional expression has been achieved in mammalian cell lines and provided information on basic spectral properties of wild type [6,7] and on spectral tuning in mutant pigments [16,19,20].

In this paper, we present the first baculovirus-mediated functional expression of human cone pigments. With this expression system larger quantities of recombinant proteins can be produced. Accordingly, purification, necessary in order to study molecular properties underlying cone pigment function, is easier to accomplish. Since histidine tagging in combination with immobilized metal affinity chromatography allows rapid and extensive purification of recombinant bovine rhodopsin [12], we constructed a 6×histidine tag at the 3' end of the synthetic human red and green cone pigment cDNAs. Both histidine-tagged pigment proteins are functionally expressed in infected insect cells at very reasonable levels ($2\text{--}4 \times 10^6$ copies/cell) under transcriptional control of very late polyhedrin pro-

Table 1
λ_{max} values (nm) for human red and green cone pigments, analyzed in vivo or through functional expression

	Red		Green
	Ser-180	Ala-180	
In vivo analysis			
MSP		558 ^a	531
SCAS		560 ^a	530
ERG	563	558	530
Functional expression			
Sf9	560	555	527
COS	563	556	532
293S	557	552	530

For the in vivo analysis values were obtained by microspectrophotometry (MSP) [22], single cell action spectra (SCAS) [23] and electroretinography (ERG) [24]. Functional expression was achieved in Sf9 cells (this study), COS cells [6,16] and 293S cells [7].

^aNo discrimination possible between the Ser-180 and Ala-180 variants in the red pigment.

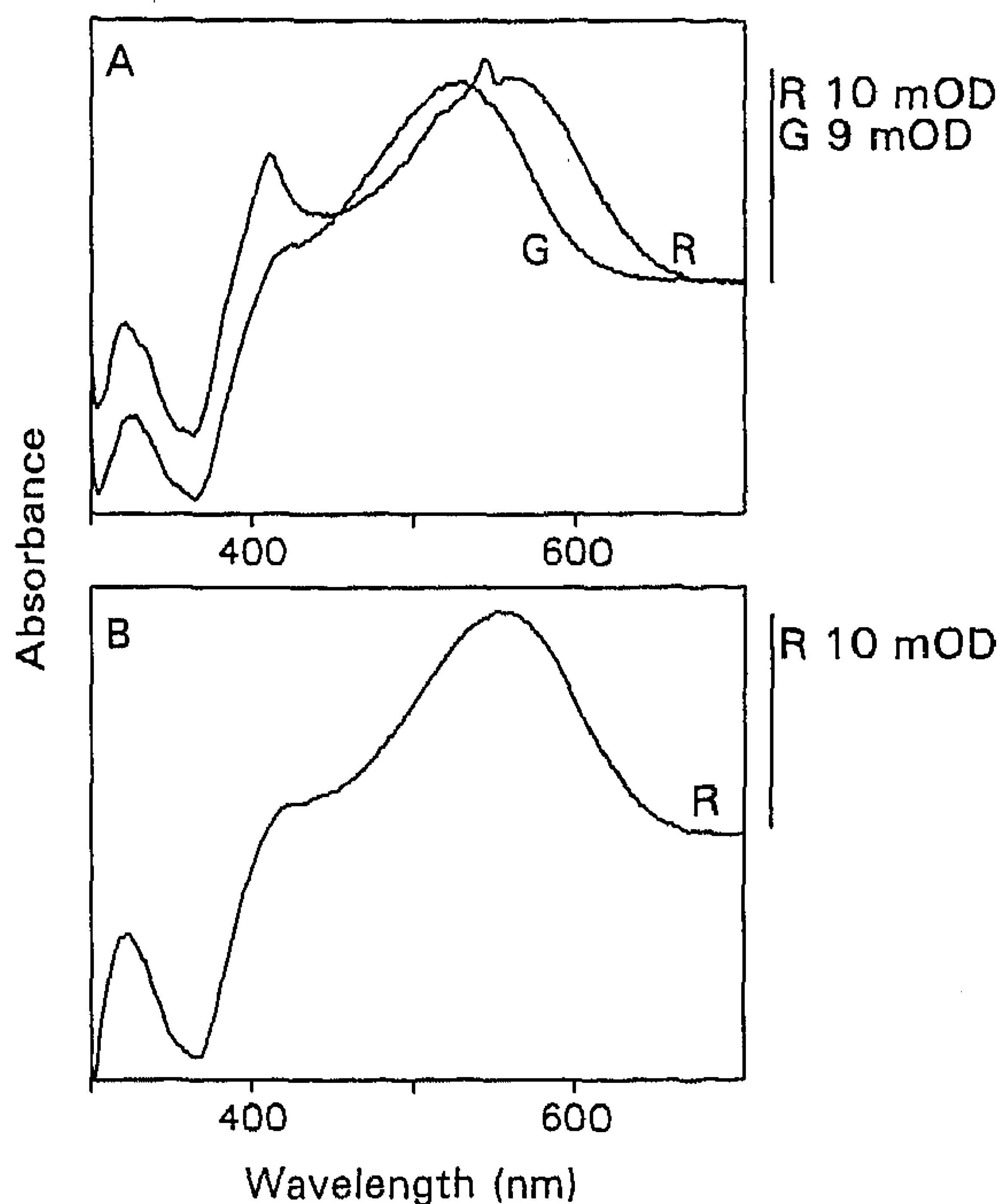


Fig. 4. Typical difference absorbance spectra of the regenerated cone pigments obtained by subtracting the spectra after illumination from those before illumination, in the presence of 20 mM hydroxylamine. A: Difference spectra of the histidine tagged red (R) and green (G) cone pigments. B: Difference spectrum of the wild type human red cone pigment. Bars at the right present the OD scale for the various spectra. The spurious peaks at 410 and 550 nm in R (A) derive from contamination by heme containing pigments.

moter. The expressed proteins can be detected by immunoblot using an N-terminally directed red-green cone pigment specific antiserum as well as a histidine tag-specific antiserum. The latter reaction clearly demonstrates that the proteins contain the histidine tag and therefore are full-length. Remarkably, earlier functional expression studies [6,7,16,19,20] did not present immunochemical identification of the cone pigments. Since the assembly of separate polypeptide fragments, corresponding to proteolytically cleaved rhodopsin, can produce a spectrally intact visual pigment [21], our results represent the first immunoblot identification of full-length mammalian cone pigments.

All sequenced vertebrate visual pigments contain one or more potential glycosylation sites in the N-terminal region. Although chicken cone pigments only have low affinity for concanavalin A-sepharose [1], this does provide evidence that these pigments are indeed glycosylated. However, no direct evidence for the presence of Asn-linked carbohydrate chains has been reported yet. The change in electrophoretic mobility observed after treatment of HGH with N-glycosidase F unequivocally demonstrates the presence of (at least) one Asn-glycan chain. The smaller shift for HGH compared to that for v-RhoHis₆× agrees with a glycosylation pattern (mono- vs. diglycosylation) based upon the difference in the number of putative glycosylation sites in the amino terminal region of these proteins (Asn-34 for HGH, Asn-2 and Asn-15 for rhodopsin). Since the human red and green cone pigments are identical in their amino-terminal region (as well as in most other intra- and extracellular domains), the red pigment will also be mono-glycosylated at Asn-34.

Confocal laser scan microscopy at 2 dpi revealed that under conditions where for the green pigment targeting to the plasma membrane can be observed, most of the red pigment is still present in the cytosol. This is probably partly due to differences in expression level. Under comparable conditions, the level of green pigment expression is approximately twice as high as that of the red pigment. The higher expression level probably induces a more rapid vesicular transport to the plasma membrane. For instance, the bovine rod pigment, of which the expression level is at least 2–3-fold higher than that of the green pigment, is largely contained in the plasma membrane ([11] and unpublished observations).

The λ_{\max} of the pigments we measure is in good agreement with other *in vitro* [6,7,16] and *in vivo* [22–24] analyses of human cone pigments (Table 1). The small differences in λ_{\max} reported for the three expression systems are within experimental error, and in our opinion there is no significant effect of the C-terminal tags on the spectral properties of the pigments. The 5 nm difference in λ_{\max} between the two red cone pigments in this study is explained by the polymorphism at position 180 (Ser in the histidine-tagged pigment, Ala in the wild type pigment). Expression in COS [16] or 293S cells [7] also resulted in a 5–7 nm red shift for the Ser-180 pigment.

Our results clearly demonstrate that the baculovirus expression system is well suited to the production of functional recombinant human cone pigments and that this can be combined with histidine tagging. Since scale-up of cell culture using the baculovirus system can be achieved relatively easily, we expect this will enable us to generate sufficient amounts of cone pigments to proceed with purification, exploiting the histidine tag. Subsequent reconstitution into a lipid environment will then allow detailed structural and biochemical analysis of mammalian cone pigments.

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