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A Fully Functional Rod Visual Pigment in a Blind Mammal

A CASE FOR ADAPTIVE FUNCTIONAL REORGANIZATION?*

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In the blind subterranean mole rat *Spalax ehrenbergi* superspecies complete ablation of the visual image-forming capability has been accompanied by an expansion of the bilateral projection from the retina to the suprachiasmatic nucleus. We have cloned the open reading frame of a visual pigment from *Spalax* that shows >90% homology with mammalian rod pigments. Baculovirus expression yields a membrane protein with all functional characteristics of a rod visual pigment (λ_{max} = 497 ± 2 nm; pK_a of meta I/meta II equilibrium = 6.5; rapid activation of transducin in the light). We not only provide evidence that this *Spalax* rod pigment is fully functional *in vitro* but also show that all requirements for a functional pigment are present *in vivo*. The physiological consequences of this unexpected finding are discussed. One attractive option is that during adaptation to a subterranean lifestyle, the visual system of this mammal has undergone mosaic reorganization, and the visual pigments have adapted to a function in circadian photoreception.

Spalax ehrenbergi represents an extreme model of a subterranean rodent, spending all its life in underground darkness with only a few occasional exits aboveground. Consequently, it displays a mosaic of reductional (regressions) and expansional (progressions) adaptation at all organizational levels. This mosaic evolution is most prominent in ocular and brain structures (1). Thirty million years of adaptation to a subterranean environment and lifestyle has resulted in a natural degeneration of the visual system of mole rats (family Spalacidae). In this process, the eye of *Spalax* has been reduced to a very small size (≤1 mm) and regressed to a subcutaneous location, embedded in a hypertrophied Harderian gland. Whereas the morphological development of the retina is normal, with a characteristic stratified organization, the anterior eye segments start to degenerate early in development (2). Neuronal components of the visual pathways are regressed or absent, and anatomically no visual cortex can be identified (3). Likewise, electrophysiological measurements did not obtain any indication for functional visual pathways (1, 4). In contrast, bilateral projections from the retina to the suprachiasmatic nucleus have been expanded (5), and *Spalax* has preserved the ability to entrain its biological clock to environmental light cues (6). Removal of the eyes abolishes circadian photoentrainment (7), demonstrating that the circadian photoreceptor system is located in the eye. Despite the degenerate nature of its visual system, *Spalax* has retained a well organized retina that expresses both a rod-like and a cone-like visual pigment. Recently a green cone-like visual pigment was cloned from *Spalax* and spectrally identified via functional expression (6), but to date only immunohistochemical evidence for the presence of a rod-like pigment has been presented (2, 8). Here we present an extensive characterization of this pigment. Its sequence is characteristic for a rod visual pigment and has retained very high homology with rod pigments of sighted mammals. Through functional expression with recombinant baculovirus we provide ample biochemical evidence that this pigment behaves like a fully functional rod pigment. Considering the absence of any visual ability, a possible novel function for the visual pigments of *Spalax* is discussed.

EXPERIMENTAL PROCEDURES

Cloning of the Spalax Rod Pigment—Total RNA was isolated from *Spalax* eyes using Trizol reagent (Life Technologies, Inc.), and approximately 2 μg of RNA was used to synthesize cDNA by means of reverse transcription (Superscript II reverse transcriptase; Life Technologies, Inc.). polymerase chain reaction amplification was performed using *Pfu* proofreading polymerase (Stratagene) in combination with degenerate nested and/or specific rod opsin primers. The degenerate and specific primers, respectively, were as follows: degenerate forward, 5′-GTG-RT-S-TGY-AAR-CCB-3′ (exon II) and 5′-AAT-GTC-GAC-CAY-GCY-ATC-ATG-GTY-3′ (exon II, nested); degenerate reverse, 5′-RTA-RAT-SAY-VGG-RTT-3′ (exon IV) and 5′-AAT-GTC-GAC-GCC-CTG-RTG-GGT-G-AA-3′ (exon IV, nested); specific forward, 5′-ATG-AAC-GGC-ACA-GA-G-GG-3′ (exon I); and specific reverse, 5′-CAT-CAC-CCA-GGA-GGT-T-CT-TGC-3′ (exon V). 5′ and 3′ rapid amplification of cDNA ends were performed using a 5′/3′ rapid amplification of cDNA ends kit (Roche Molecular Biochemicals) in combination with an opsin-specific reverse primer in exon I (5′-AAG-TTG-AGC-AGG-ATG-TAG-3′) and the above-mentioned forward-nested degenerate rod opsin primer in exon II, respectively. The obtained full-length open reading frame was checked on the genomic level by means of the expanded long template polymerase chain reaction system (Roche Molecular Biochemicals) using 500 ng of genomic DNA and a rod opsin-specific primer (5′-TCT-ACG-TGC-CCT-TCT-CCA-ACG-3′) in exon I in combination with the above-mentioned specific rod opsin reverse primer in exon V. The obtained partial sequence was identical to the corresponding cDNA sequence. A forward primer with a *Bam*HI site (5′-GGC-GGG-ATC-CAT-GAA-CGG-CA-3′) and a reverse primer containing a *Nar*I site (5′-TTA-

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GGC-CGG-CGC-CAC-CTG-G-3′) were used in combination with the *Pfu* proofreading polymerase to clone the *Spalax* rod opsin cDNA in a baculovirus transfer vector modified from pFastbacdual (Life Technologies, Inc.). This modified transfer vector contains a *Bam*HI site and a *Nar*I site in frame with the remaining 3′ part of the *Spalax* rod opsin sequence extended with an oligonucleotide coding for 6 histidines (His-tag).

Functional Expression—The transfer vector containing the His-tagged *Spalax* rod opsin cDNA was used to generate recombinant baculovirus in a *Spodoptera frugiperda*-derived cell line (IPLB-Sf9) using the Bac-to-bac system (Life Technologies, Inc.) according to manufacturer's instructions. Generation and amplification of the recombinant baculovirus was performed in a monolayer of Sf9 cells, cultured in TNM-FH medium supplemented with 10% (v/v) fetal calf serum, 50 units/ml penicillin, and 50 μg/ml streptomycin (Life Technologies, Inc.). For expression of recombinant protein (9) Sf9 cells were adapted to suspension culture in 250-ml spinner flasks (Bellco) in serum-free InsectXpress medium (Biowhittaker), infected in mid log phase with a multiplicity of infection of 0.1, and finally harvested 5 days post-infection.

Regeneration with 11-cis Retinal, Purification, and Reconstitution of Recombinant Pigment—At 5 days post-infection the infected Sf9 cells were harvested by centrifugation (10 min; 1000 $\times$ *g* at 26 °C) and resuspended at a concentration of 10^8 cells/ml in buffer A (6 mM Pipes,[1] 10 mM EDTA, pH 6.5), supplemented with 5 mM β-mercaptoethanol and 2 μg/ml leupeptin. The suspension was homogenized in a Potter-Elvehjem homogenizer and centrifuged (10 min; 40,000 $\times$ *g* at 4 °C). The pellet containing the cellular membranes was resuspended at a concentration equivalent to 10^8 cells/ml in buffer B (20 mM Pipes, 130 mM NaCl, 10 mM KCl, 3 mM $MgCl_2$, 2 mM $CaCl_2$, 0.1 mM EDTA, 2 μg/ml leupeptin, pH 6.5). All subsequent manipulations are performed under deep red light (Schott R630 longpass filter). Regeneration of the recombinant *Spalax* opsin into rhodopsin was performed under argon by addition of a concentrated 11-*cis* retinal solution in dimethylformamide to the membrane suspension in an amount of 10 nmol/10^8 cells. The mixture was incubated under continuous rotation for 30 min at room temperature followed by 1 h at 4 °C. To extract the rhodopsin from the membranes *N*-dodecyl-β-D-maltoside was added to a final concentration of 1% (w/v), and the resulting mixture was incubated for 1 h under argon with continuous rotation at 4 °C. After centrifugation (30 min; 120,000 $\times$ *g* at 4 °C) the supernatant was diluted 1:1 with buffer C (20 mM bis-Tris propane, 0.5 M NaCl, 20% glycerol (v/v), 5 mM β-mercaptoethanol, 1 mM histidine, 2 μg/ml leupeptin, 40 mM *N*-nonyl-β-D-glucoside, pH 7.0).

The solubilized pigment was purified by immobilized metal affinity chromatography over Ni^{2+} nitrilotriacetic acid resin (Qiagen) in a slight modification from previously described procedures (9, 10). Prior to purification the pigment concentration in the extract was determined by UV-visible difference spectroscopy (10), and the pH value was raised to 7.0–7.2 by addition of several aliquots of 200 mM unbuffered bis-Tris propane solution. The Ni^{2+} nitrilotriacetic acid resin (25–30 μl of resin/nmol of pigment) was washed with 10 volumes of distilled water and buffer C, respectively. The protein extract was then applied to the column and washed with approximately 10 column volumes of buffer C followed by approximately 10 column volumes of a linear gradient prepared from buffers C and D (buffer D, buffer C with the histidine concentration raised to 5 mM and containing 20 mM *N*-nonyl-β-D-glucoside). Elution was accomplished using buffer E (20 mM bis-Tris propane, 140 mM NaCl, 20% glycerol (v/v), 20 mM *N*-nonyl-β-D-glucoside, 5 mM β-mercaptoethanol, 50 mM histidine, 2 μg/ml leupeptin, pH 6.5). Fractions were screened by UV-visible spectroscopy, and those containing over 0.5 nmol of rhodopsin/ml were combined.

To reconstitute the purified pigment into a lipid membrane (proteoliposomes) a 100-fold molar excess of bovine retina lipids in buffer F (20 mM Pipes, 130 mM NaCl, 15 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.1 mM EDTA, 2 μg/ml leupeptin, pH 6.5) supplemented with 20 mM *N*-nonyl-β-D-glucoside and 1 mM dithioerythritol was added to the combined rhodopsin fractions. Preparation of bovine retina lipids and removal of the detergent through addition of β-cyclodextrin to a final concentration of 20 mM were performed as described before (11). Purification of the proteoliposomes containing the reconstituted pigment was accomplished on a discontinuous sucrose gradient (10, 20, and 45% sucrose (w/w)) in buffer F (11). After 16 h of centrifugation at 120,000 $\times$ *g* and 4 °C the pigment containing proteoliposomes were isolated from the 20–45% interface and diluted with 4 volumes of buffer B. The membranes were then pelleted (200,000 $\times$ *g*, 30 min at 4 °C) and stored at −80 °C in light-tight containers.

For spectral analysis of the pigment a PerkinElmer Life Sciences Lambda 15 spectrophotometer was used with the cuvette house thermostrated at 10 °C. Membrane samples were used for analysis of the slow photocascade transitions (rhodopsin → meta II → meta III) as described before (12). Absorbance spectra were measured after solubilization in buffer B containing 20 mM *N*-dodecyl-β-D-maltoside. The samples were supplemented with hydroxylamine to a final concentration of 20 mM and were measured before and after illumination with a 75-watt light bulb. Difference spectra were obtained by subtraction of the "dark" from the "illuminated" spectrum.

Transducin Activation Assay—Activation of the bovine G-protein transducin (G_t) by the *Spalax* rod pigment was measured by the intrinsic fluorescence enhancement of the G-protein α subunit upon GTP binding (13). Native bovine rhodopsin or reconstituted pigment samples (~20 μl) of recombinant His-tagged *Spalax* or bovine rhodopsin (final concentration of 5 nM) were added under illumination to a stirred cuvette containing, in a final volume of 2 ml, 100 nM bovine transducin, 100 mM NaCl, 2 mM $MgCl_2$, 1 mM dithioerythritol, 0.01% (w/v) *N*-dodecyl-β-D-maltoside, 20 mM Hepes, pH 7.4. The sample fluorescence was measured using a Shimadzu RF—5301–PC spectrofluorometer with excitation at 295 nm and emission at 337 nm, and after a stabilization period of 200 s, guanosine 5′-*O*-(3-thiotriphosphate) was added to a final concentration of 2.5 μM. The subsequent increase in relative fluorescence intensity represents pigment-triggered activation of $G_t\alpha$. The same assay in the absence of pigment was used as a negative control. Constitutive activity of the apoprotein opsin was assayed in the same way, except that the pigment samples were illuminated in the presence of 50 mM hydroxylamine to convert photointermediates and retinal into opsin and retinaloxime before they were added to the transducin solution.

To measure any dark activity of the pigment, the fluorescence enhancement assay was used, as well, but was modified as follows: about 25 ml of a solution containing dark pigment, transducin, and the other compounds in the same composition as given above was prepared under deep red light. Guanosine 5′-*O*-(3-thiotriphosphate) was then added to a final concentration of 2.5 μM, and at subsequent 10-min intervals 2-ml samples were withdrawn. After 80–90 min the remaining solution was illuminated, and subsequently 2-ml samples were taken every minute to measure the light activation level. Every 2-ml sample was provided immediately with 50 μl of 1 M hydroxylamine (pH 7.5) and 50 μl of 25 mM *N*-dodecyl-β-D-maltoside to quench any ongoing and latent G-protein activation activity. Control experiments showed that under these conditions G-protein activation by light-activated rhodopsin is quenched below 1% its normal rate. The samples were illuminated, and their fluorescence level was measured as described above for the continuous assay. This level was stable for at least 4 min.

Microspectrophotometry—Spalax eyes were isolated from dark-adapted animals and the retina was excised and mounted between coverslips in the microscope, and potential outer segment material was identified under infrared illumination. The spectral transmission of the photoreceptor outer segments was scanned using a 2-μm diameter beam of monochromatic light over the wavelength range of 350 to 750 nm (14). This resulted in the dark spectrum. Following a 3-min exposure to white light this procedure was repeated to establish whether the pigment is photosensitive. The dark spectrum was fitted with standard Dartnall templates for a vitamin A1-based visual pigment (14).

Immunohistological Analysis—Polyclonal antibodies CERN886 against rhodopsin and CERN9412 against $G_t\alpha$ were described previously (15). Monoclonal antibodies against rhodopsin (1D4) or against $G_t\alpha$ were obtained from R. S. Molday (16) and A. M. Spiegel (17), respectively.

Spalax ocular tissue was fixed in 4% paraformaldehyde/PBS and mouse ocular tissue in Bouin fixative (3 volumes saturated picrine acid, 1 volume 37% formaldehyde, and 0.2 volume of acetic acid). Paraffin-embedded 4-μm sections were used for antibody incubations and hematoxylin/eosin staining. *Spalax* and mouse ocular tissue were incubated according to standard procedures with 1D4 or anti-$G_t\alpha$ (dilutions 1:50 and 1:100 in PBS/10% fetal calf serum, respectively) and with CERN886 or CERN9412 (dilutions 1:100 and 1:500 in PBS/10% fetal calf serum, respectively).

Bound antibody was visualized by incubation with a fluorescent second antibody (fluorescein isothiocyanate-rabit anti-mouse (DAKO) for 1D4 and anti-$G_t\alpha$, fluorescein isothiocyanate-goat anti-rabbit (DAKO) for CERN886 and CERN9412; dilutions 1:50 in PBS/10% fetal

[1] The abbreviations used are: Pipes, 1,4-piperazinediethanesulfonic acid; G_t, G-protein transducin; PBS, phosphate-buffered saline; bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.

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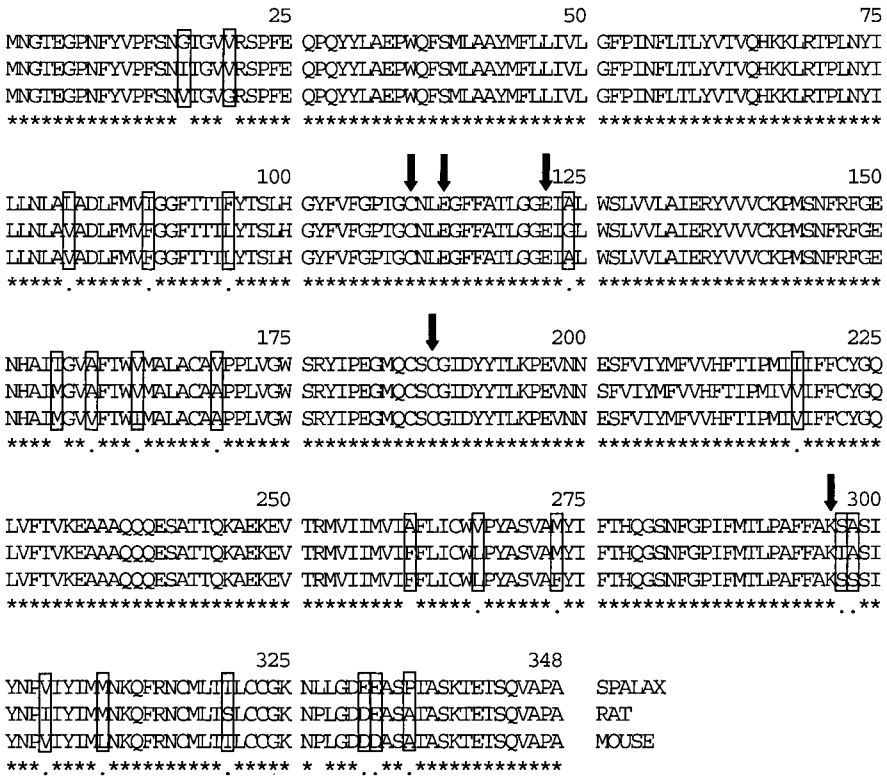

FIG. 1. **Multiple sequence alignment of the amino acid sequence of *Spalax*, rat, and mouse rhodopsin.** The nucleotide sequence of *Spalax* rhodopsin cDNA contains an open reading frame that codes for 348 amino acid proteins and shares approximately 95% similarity with other rodent rhodopsins like rat and mouse. The amino acid substitutions in *Spalax* compared with rat (16 in total) and mouse (19 in total) rhodopsin are *boxed*. Note (*arrows*) the presence in *Spalax* of the over all vertebrate visual pigments fully conserved residues Lys-296 (11-*cis* retinal binding), Glu-113 (counterion), and Cys-110 and Cys-187 (disulfide bridge). The *fifth arrow* indicates the Glu residue at position 122, highly characteristic for rod visual pigments. The *Spalax* sequence is available under GenBand™ acession number AF309568.

calf serum). Coverslips were mounted using a ProLong anti-fade kit (Molecular Probes) according to the manufacturer's instructions. Fluorescence microscopy was performed on a Zeiss Axioskop microscope.

RESULTS AND DISCUSSION

Structural and Functional Characterization of the Spalax Rod Visual Pigment in Vitro—To clone the rod pigment from *Spalax* retina we conducted RT polymerase chain reaction and 5′ and 3′ rapid amplification of cDNA ends on total ocular RNA using degenerate and rhodopsin-specific primers. The amino acid sequence deduced from the full-length cDNA displays a very high similarity with other mammalian rhodopsins (91–95%), and all elements essential for a functional visual pigment are conserved (Fig. 1).

To establish whether the isolated cDNA encodes for a functional rod pigment we extended the cDNA C-terminally with a 6× His-tag to allow easy purification and expressed it using the baculovirus system. After regeneration with the chromophore, 11-*cis* retinal, purification was accomplished using immobilized metal affinity chromatography (10). This yields a purified protein that has an apparent molecular mass of 40 kDa (Fig. 2) and an absorbance spectrum with a peak spectral activity of 497 ± 2 nm (Fig. 3). As expected, the spectral properties are very similar to those of rod pigments in sighted animals, because all known spectral tuning sites (18) have been conserved in *Spalax*. However, immunoblots of *Spalax* eye extracts suggest a higher molecular mass of ~43 kDa (Fig. 2). Because the protein component of the *Spalax* and bovine rod pigments are very similar (39 kDa), and the recombinant pigments display the same molecular mass (Fig. 2), most likely the native *Spalax* pigment contains larger oligosaccharide moieties on its two *N*-glycosylation sites, which will reduce its electrophoretic mobility. The very small *Spalax* eye only contains minute amounts of rhodopsin (~5 pmol (8)), which so far has prohibited isolation and structural characterization of the native protein.

To study the pigment's photocascade and receptor activity in a native-like environment, we reconstituted the pigment into proteoliposomes of bovine retina lipids (11). The reconstituted

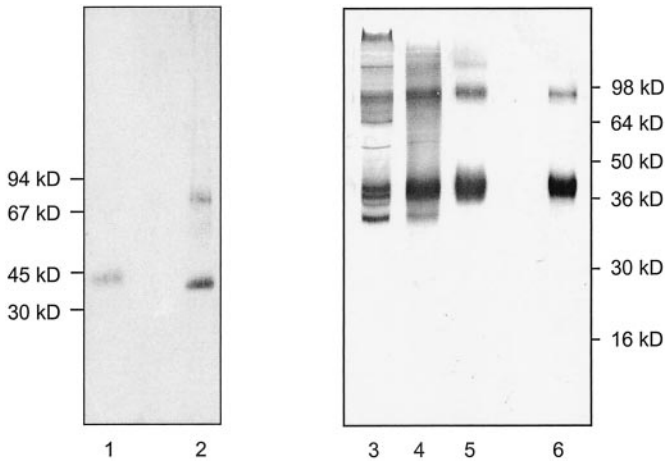

FIG. 2. **Western blot analysis of rhodopsin preparations using CERN886 anti-rod polyclonal antibody (dilution 1:1000).** *Left panel*, *Spalax* eye extract (*lane 1*) and mouse eye extract (*lane 2*). *Right panel*, Sf9 cells expressing His-tagged *Spalax* rhodopsin (*lane 3*); *N*-dodecyl-β-D-maltoside extract of Sf9 cells (*lane 4*); purified and reconstituted His-tagged *Spalax* rhodopsin (*lane 5*); purified and reconstituted His-tagged bovine rhodopsin (*lane 6*). Note the apparent higher molecular mass of native *Spalax* rhodopsin (*lane 1*, 43 kDa) relative to mouse (*lane 2*, 40 kDa).

Spalax rhodopsin displays typical rod pigment photochemical behavior (pK_a of meta I/meta II equilibrium of 6.5; half-time of meta II decay of 15 ± 3 min at 10 °C). *Spalax* is very similar to the human rod pigment in this respect (19). Note that the decay of meta II is typically much faster in cone pigments ($t_{1/2} < 3$ min (20)). The structural transitions accompanying receptor activation were probed by fourier transform-infared difference spectroscopy and were found to be almost identical to recombinant His-tagged bovine rhodopsin (not shown). This already implies that the *Spalax* rod pigment should be able to activate the rod G-protein transducin. Indeed, in an *in vitro* assay (13) it achieves a light-induced activation rate very similar to that of

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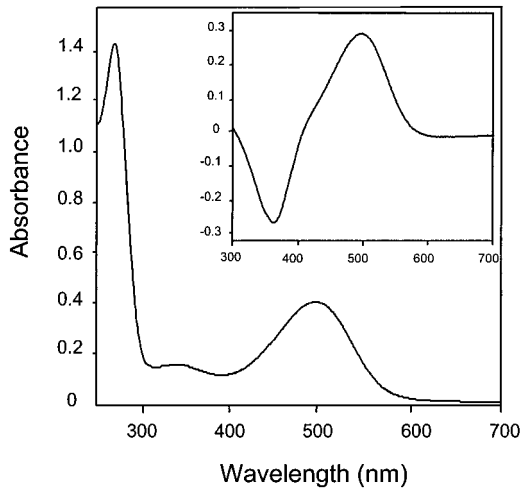

FIG. 3. **Spectral properties of purified reconstituted *Spalax* rhodopsin.** The photopigment displays a peak spectral activity at 497 ± 2 nm. The A_{280}/A_{500} ratio is approximately 3.5. This is higher then for purified rod pigments (1.7–2.0) because of the excess of lipid that contributes to the UV absorbance. The *inset* shows the difference spectrum, obtained by subtracting the dark spectrum from the spectrum after illumination. The spectra were measured in the presence of 20 mM hydroxylamine to convert the liberated all-*trans* retinal into all-*trans* retinoxime with an absorbance maximum at 365 nm.

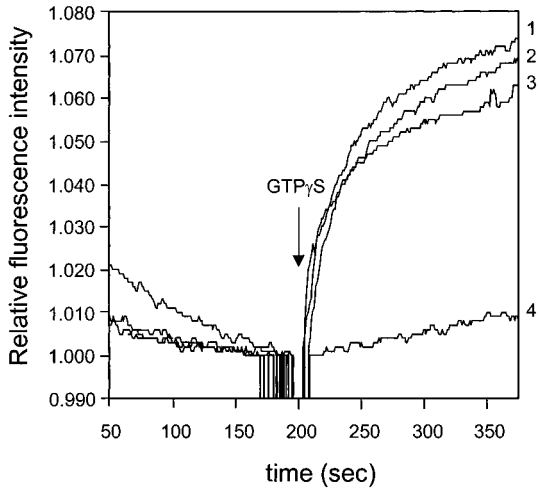

FIG. 4. **Light-dependent activation of bovine G_t by the *Spalax* rod pigment.** Intrinsic fluorescence enhancement of the G-protein α subunit upon GTP binding was used to monitor activation by either native bovine rhodopsin (*trace 3*) or recombinant His-tagged *Spalax* (*trace 1*) or bovine (*trace 2*) rhodopsin. As a negative control the activation rate in the absence of pigment was measured (*trace 4*). All pigments were present at the same concentration (5 nM), and the reaction was initiated by the addition of guanosine 5′-*O*-(3-thiotriphosphate) (*GTPγS*) to 2.5 μM (*arrow*).

native and of recombinant bovine rhodopsin (see Fig. 4 and Table I). The dark activity of the *Spalax* pigment is very low, and the constitutive activity of the apoprotein opsin is quite low, as well (Table I), very similar to the properties reported for bovine rhodopsin (Table I) (13). We therefore conclude that in vitro the Spalax rod photopigment behaves as a fully functional visual pigment.

Functional Characterization of the Spalax Rod Pigment in Vivo—Do we have evidence for a functional pigment *in vivo*? Both 11-*cis* retinal (8) and a functional interphotoreceptor retinoid binding protein (21) have been identified in *Spalax* retina, suggesting a functional visual cycle. Therefore, we attempted to identify a photopigment *in vivo* by means of microspectrophotometry. Despite a much lower degree of organization of *Spalax* photoreceptor outer segments we managed to determine the spectral absorbance of several outer segments in retinas isolated from dark-adapted animals (Fig. 5). The corresponding pigment is photosensitive, and its absorbance curve obeys the Dartnall standard template for a vitamin A1-based visual pigment (14). The dashed curve (Fig. 5) represents a template with a peak absorbance at 497 nm, as determined for the recombinant pigment. This template fit is not optimal, which could be because of the noise level in the spectral data or might indicate that *in vivo* the absorbance of the pigment is slightly shifted to the red. Such small discrepancies between spectral data of native and recombinant pigments have been observed before (18). Nevertheless, the close agreement of the *in vivo* spectrum with our *in vitro* data strongly argues that the *Spalax* retina contains a photoactive rod pigment.

TABLE I
Transducin (G_t) activation by Spalax rhodopsin relative to bovine rhodopsin

The light-dependent initial activation rate measured for native bovine rhodopsin was set at 1 (see Fig. 4). n = 3–5. N.A., not applicable.

Preparation	Condition	Initial rate
Recombinant his-tagged bovine rhodopsin	dark	0.01 ± 0.01
	light	0.97 ± 0.05
Recombinant his-tagged *Spalax* rhodopsin	dark	0.01 ± 0.01
	light	0.95 ± 0.06
Recombinant his-tagged *Spalax* opsin	N.A.	0.05 ± 0.03

A functional visual pigment requires co-localization with the rod G_t. To address that question, we performed immunohistochemical analysis of *Spalax* retina with anti-rhodopsin and anti-$G_t\alpha$ antibodies. We observed abundant expression of rod pigment in *Spalax* photoreceptor outer segments and clear staining of inner segments and the outer nuclear layer (Fig. 6*C*). This is similar to the picture obtained for mice (Fig. 6*D*). $G_t\alpha$ expression was observed in *Spalax* but only in the outer segment layer (Fig. 6*E*) in contrast to mouse where $G_t\alpha$ is detected throughout the entire photoreceptor cell (Fig. 6*F*). This could be because of a lower avidity of the antiserum for *Spalax* $G_t\alpha$. However, the same pattern was observed with different dilutions of the antiserum and with the monoclonal anti-$G_t\alpha$ antibody raised against a highly conserved sequence of $G_t\alpha$ (17). Rather this suggests that in *Spalax* the expression level of $G_t\alpha$ relative to rhodopsin is lower as compared with mouse. This might be related to the subterranean environment of the mole rat. For instance, to guarantee maximal photon capture under the very low light intensities experienced by *Spalax*, the high expression level of visual pigment, typical for sighted animals, should be maintained. Nevertheless, the level of activated pigment will be very low, and signal transduction should be able to operate with much lower G_t levels than in sighted animals. Considering our *in vitro* data (spectral properties and full activation of G_t) and the *in vivo* evidence for the same photosensitive pigment co-localized with $G_t\alpha$, we conclude that the *Spalax* rod visual pigment is functional *in vivo* and uses the same photocascade as sighted animals.

A Functional Photopigment in a Blind Mammal: Rudiment or Mosaic Reorganization?—Although visual pigments have been identified before in blind animals like the cave fish *Astyanax* (22) and crayfish (23), we here report the first extensive functional characterization of a visual pigment in a blind mammal. In other blind animals loss of sight seems to be accompanied by deficiencies in gene expression or pigment functionality (22–24). In contrast, the rod visual pigment of the blind mole rat we have cloned is highly conserved and exhibits full functionality.

A major question then is why a blind mammal, despite mo-

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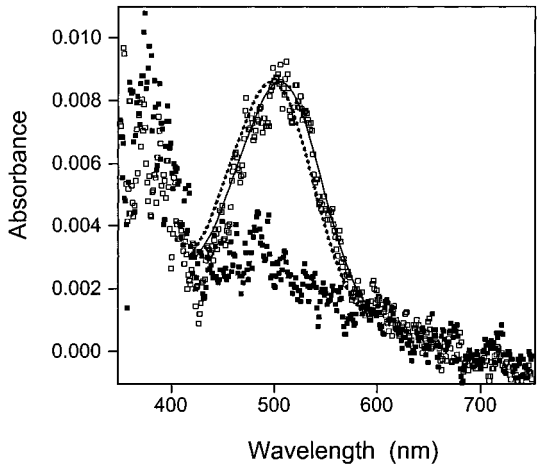

FIG. 5. **Spectral absorbance data from four *Spalax* photoreceptor outer segments obtained by microspectrophotometry.** *Open squares* were measured before illumination, and *solid squares* were measured after 3 min of illumination with white light. The *dashed curve* represents a visual template (14) with a λ_{max} of 497 nm. This yields a reasonable fit, but better fits are exhibited by 500- to 505-nm templates. The 505-nm template is represented by the *solid curve*.

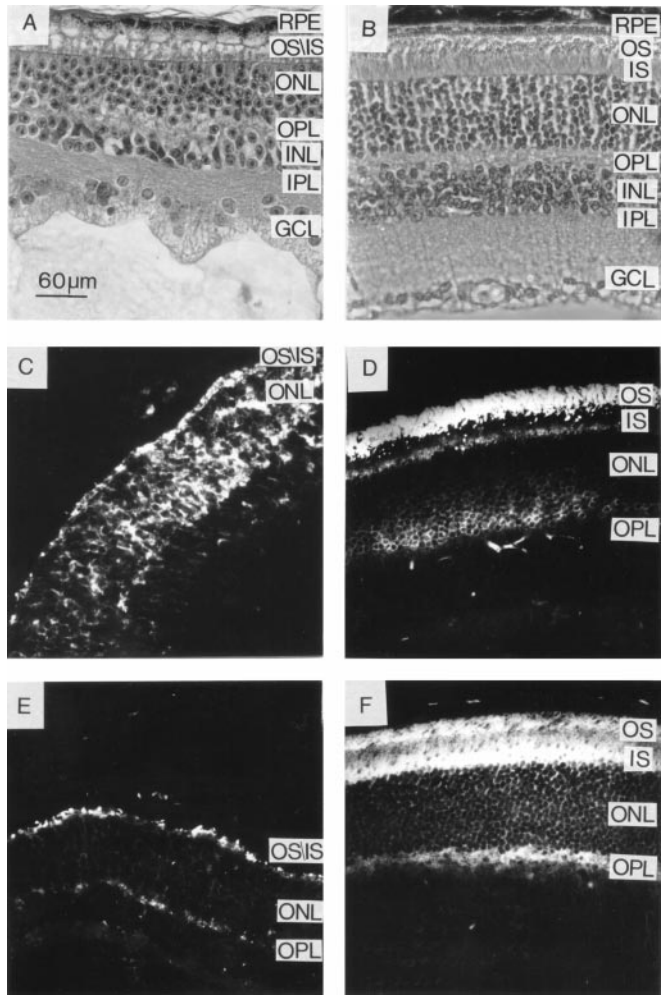

FIG. 6. **Rhodopsin and $G_t\alpha$ localization in *Spalax* and mouse retina.** *A* and *B* display the retina morphology of *Spalax* and C57BL6 mouse using a hematoxylin/eosin staining. *C–F* display immunohistological analysis of *Spalax* and mouse retina incubated with the monoclonal anti-rhodopsin antibody 1D4 (*C* and *D*) and with the polyclonal anti-G_t antibody CERN9412 (*E* and *F*), respectively. Results identical to *C* and *D* were obtained with the polyclonal anti-rhodopsin antibody CERN886 and to *E* and *F* with a monoclonal anti-$G_t\alpha$ antibody. *RPE*, retina pigment epithelium; *OS*, outer segments; *IS*, inner segments; *ONL*, outer nuclear layer; *OPL*, outer plexiform layer; *INL*, inner nuclear layer; *IPL*, inner plexiform layer; *GCL*, ganglion cell layer. *Bar* represents 60 μm for all *panels*.

saic reorganization of visual structures to an auditory function (1, 3, 5), has retained a fully functional visual pigment? The subcutaneous location of the eye and the complete degeneration of the lens (2) already abolish any capacity of the retina to transfer image information. In fact, all available evidence, based upon anatomical, electrophysiological, and behavioral studies, convincingly shows that the visual pathways in *Spalax* have almost fully regressed and do not retain any detectable function (1, 3–5, 7). Ocular regression to a subcutaneously embedded atrophic eye and degeneration of the visual system is a logical consequence of adaptation to a subterranean environment where visual cues are reduced. However, negative selection upon the neuronal components of the visual system was accompanied by positive selection pressures resulting in maintenance of retinal morphology and expansion of bilateral projections to the suprachiasmatic nucleus. This pathway is responsible for mediating circadian photoentrainment (1, 4, 5), and, similar to sighted mammals, removal of the eyes completely abolishes photoentrainment in *Spalax*.

In sighted mammals the photoreceptors that mediate photoregulation of the circadian system still remain uncharacterized. Data obtained from mouse models like *rd/rd* and *rdta/rdta* (25–27) suggest that neither rods nor cones are directly required for photoentrainment but that the mouse retina contains a novel type of circadian photoreceptor(s), not located in the photoreceptor layer. However, the circadian system in sighted animals is not very photosensitive (28, 29) and may not function properly in combination with a subterranean lifestyle. Hence, the crucial question in our opinion is as follows: did *Spalax* maintain a photoreceptor cell layer and functional visual photopigments basically to preserve circadian photoreception? In fact, in blind animals like *Spalax*, maintenance of visual photoreceptors without any physiological importance would go with high metabolic costs and appear to contradict evolutionary laws (1).

Considering such admittedly circumstantial evidence we propose that *Spalax*, in its adaptation to a blind subterranean life, has rewired its retinal circuitry to maintain circadian photoentrainment under conditions where light cues are sparse and of low intensity. In this view, extensive deprivation of light because of a subterranean ecotope and the subcutaneous location of the eyes have expanded mosaic reorganization in *Spalax* to complement its "traditional" non-visual photoreceptor circadian system with the much more abundant and much more sensitive visual photoreceptor system, which could be diverted from its original function. This demanded that the full integrity of the retinal morphology be maintained but without the need for an optical focusing system (cornea and lens). Direct evidence for this hypothesis unfortunately is very hard to provide. The *Spalax* underground ecotope cannot very well be mimicked, and animals do not breed in captivity. Hence, recombinant DNA technology that could lead to identification of circadian pigment(s) (*e.g.* mutagenic screens and gene targeting) is not currently available for *Spalax*. Determination of sufficiently accurate action spectra for the circadian photoresponses in *Spalax* to provide unequivocal evidence for the involvement of visual pigments is also not a feasible task (6).

However, another circumstantial argument can be put forward. The degenerate eye of *Spalax* shares several characteristics with the pineal organ of non-mammalian vertebrates. Both lack a focusing lens, are located subcutaneously, and exhibit a much lower degree of organization of the outer segments of the photoreceptor cells than observed for visual pho-

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toreceptors (28, 30–32). The pineal photoreceptors also contain rod-like, as well as cone-like, photopigments (31, 33) and have unequivocally been shown to function as photodetectors mediating photoperiodic information in non-mammalian vertebrates (28, 32, 34, 35).

Conclusion—We present the first extensive characterization of a visual pigment cloned from a blind animal, the mole rat *Spalax ehrenbergi*. In view of its high homology to rod pigments of sighted animals and the functional properties of the corresponding recombinant protein, this pigment represents the rod visual pigment of *Spalax*. We provide evidence that the rod photoreceptor cell of *Spalax*, despite the strongly regressed state of the eye, still contains the primary elements required for a role as a functional photoreceptor. In view of the striking evolutionary conservation and the *in vitro* and *in vivo* functionality of the *Spalax* rod visual pigment we propose that during evolutionary adaptation to a predominantly subterranean lifestyle the visual photoreceptor system in this blind mammal has been reprogrammed to play a major role in photoperiodic entrainment. Extrapolating this to sighted animals, one could argue that this might imply some yet unidentified role, *e.g.* developmental or regulatory, of the visual system in circadian photoentrainment in general. This might explain, for instance, why early ablation of photoreceptors during retinal development changes the photosensitivity of the circadian system (25).

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A Fully Functional Rod Visual Pigment in a Blind Mammal: A CASE FOR ADAPTIVE FUNCTIONAL REORGANIZATION?

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