A C-terminal truncation results in high-level expression of the functional photoreceptor sensory rhodopsin I in the archaeon *Halobacterium salinarium*

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Summary

Expression of the gene encoding the halobacterial photoreceptor sensory rhodopsin I (SRI), *sopl*, was studied by means of homologous gene targeting. A *sopl*⁻ *Halobacterium salinarium* mutant strain was constructed by homologous replacement of *sopl* with a novobiocin-resistant *gyrB* from *Haloferax* Aa 2.2. Cells bearing *gyrB* were resistant to novobiocin, indicating that the *Haloferax* gene is functional in *H. salinarium*. Complementation of this deletion strain with *sopl* fused to the bacterio-opsin promoter resulted in the recovery of all phenotypical attributes of SRI. This establishes the first direct correlation between *sopl* and the function of its gene product. In the complemented deletion strain, functional expression of *sopl* occurred from the *bop* locus, where *sopl* had integrated by homologous recombination. This shows that cotranscription of *sopl* and the gene encoding the SRI signal transducer, *htrI*, which is found in the wild type, is not a prerequisite for photosensory activity. Deletion of the last 43 bp at the 3' end of *sopl* resulted in a 10-fold increase in the amount of SRI, without affecting the activity of the pigment. The mRNA level of the truncated gene was not affected as compared to that of the wild type. We propose that regulation occurs at the protein level, probably through a negative determinant of protein stability located in the C-terminus of SRI. Replacement of the last 28 amino acids of bacteriorhodopsin by the last 29 amino acids of SRI results in a decrease of the bacteriorhodopsin, supporting our observations. The C-terminus of SRI is the first domain with a downregulating influence on protein levels thus far identified in *H. salinarium*. The system for SRI overexpression we present here greatly facilitates biochemical and biophysical studies on the photoreceptor and allows

investigation of the molecular interactions underlying the signal transduction chain of halobacterial phototaxis.

Introduction

Retinal proteins are a group of membrane proteins which possess the ability to interact with light and to decode its information. Their structure is strongly conserved and consists of seven membrane-spanning hydrophobic α -helices and a covalently linked retinal chromophore. In Eucarya, retinal proteins function as visual photoreceptors. In the archaeal species *Halobacterium salinarium* (formerly called *H. halobium*) they provide both the conversion of light energy into an ionic electrochemical potential (photosynthesis) as well as the detection of spatial and temporal variations in light intensity and colour (phototaxis). The halobacterial retinal pigments dedicated to photosynthesis are the ion pumps bacteriorhodopsin (BR) and halorhodopsin (HR) (for a review see Oesterhelt *et al.*, 1992). Phototactic reception is mediated by the sensory rhodopsins I and II (SRI and SRII) (for a review see Bogomolni and Spudich, 1991). SRII is a purely photophobic receptor with an action spectrum maximum at 480 nm, while SRI occurs in two photoactive forms: it is an attractant receptor for orange light ($\lambda_{\text{max}} = 587$ nm) and a repellent receptor for blue light ($\lambda_{\text{max}} = 373$ nm). Under constant illumination, the two SRI species are present as a photochromic mixture. SRI, therefore, confers on halobacteria a simple colour-discriminating ability which enables the cells to concentrate in regions of light which are optimal for energy capture by BR and HR.

Significantly, the synthesis of SRI is regulated via the same environmental stimuli as BR and HR (Otomo *et al.*, 1988); expression of their respective genes *sopl*, *bop*, and *hop* is strongly induced in the late stationary phase under conditions of low oxygen tension and strong illumination. The relative cellular contents of BR, HR and SRI, however, are completely different; the molar ratio in the cell membrane is 100:10:1, respectively. In wild-type *H. salinarium*, there are only 2000–3000 copies of SRI present in a cell (Otomo *et al.*, 1988). The purification of SRI from *Halobacterium* membranes is, therefore, a tedious procedure which involves detergent and yields the protein in a high molar excess of exogenous retinal needed for its

Fig. 1. Construction of the *sopI* replacement vector pBB (see the Experimental procedures for details). Ap^R ampicillin resistance; Nov^R novobiocin resistance

stabilization (Schegk and Oesterhelt, 1988). The low natural abundance of SRI in *Halobacterium* has been the major hindrance to its thorough biochemical and spectroscopic characterization. Thus, one of our goals was to enhance the amount of SRI in *H. salinarium* cells. To this purpose we took advantage of the recent progress in the field of halobacterial molecular genetics and employed homologous gene targeting as a tool to study *sopI* expression. This method is based on the recombination between homologous DNA in a plasmid vector and genomic DNA, a process that has been shown to occur efficiently in *H. salinarium* (Mankin *et al.*, 1992; Ferrando *et al.*, 1993). A genetically defined SRI-negative *Halobacterium* strain was obtained, and the SRI deficiency was then complemented with specifically engineered *sopI* expression cartridges which integrate into the genome. This expression system allows for structure-function studies on the photoreceptor itself by site-directed mutagenesis. Moreover, it permits the analysis of the interaction of SRI with further components of the signal transduction chain of phototaxis, in particular the recently

identified methyl-accepting transducer HTRI (halobacterial transducer rhodopsin) (Yao and Spudich, 1992; Ferrando-May *et al.*, 1993). Finally, we present results which provide evidence that the C-terminus of SRI is involved in the control of the abundance of this protein, but not in its function.

Results

Targeted replacement of *sopI*

Our strategy for the specific elimination of SRI synthesis in *H. salinarium* consisted in the replacement of the *sopI* coding sequence on the genome via homologous recombination. As a selectable marker we employed a mutant *gyrB* gene from the halobacterial genus *Haloferax* Aa 2.2 which confers resistance to novobiocin (Holmes *et al.*, 1991). In the gene-targeting vector pBB, which carries a 5.8 kb genomic fragment of *H. salinarium* (see Fig. 1 and the Experimental procedures), the coding sequence of *sopI* was replaced by *gyrB*. pBB does not contain any halobacterial origin of replication. Stable transformation to

Fig. 2. Targeted replacement of *sopI* in the *H. salinarium* genome. A. Diagram of the homologous recombination of the novobiocin resistant *gyrB* at the *sopI* locus via a double cross-over event. B. Southern analysis of genomic DNA from the *sopI*⁻ deletion strain LSD3 and the parent strain L33. In the DNA of L33, the *gyrB* probe identifies two cross-hybridizing fragments from the endogenous DNA gyrase gene. C. PCR of genomic DNA from the strains LSD3 and L33 using the oligonucleotides depicted in (A).

Fig.3. Western blot of SRI-containing membranes. Lanes: A, *sopl* wild-type strain L33; B, *sopl*⁻ deletion strain LSD3; C, strain M22 expressing *sopl* under control of the *bop* promoter. Each lane was loaded with 20 µg total membrane protein.

novobiocin resistance therefore required the incorporation of the mutated *gyrB* gene into the chromosome; the adjacent homologous sequences directed this incorporation to the *sopl* locus. Transformation of the *H. salinarium* strain L33 with pBB DNA yielded colonies resistant to novobiocin, showing that *gyrB* from *Haloferax* is successfully expressed in *H. salinarium*. About 55% of the total novobiocin-resistant transformants displayed the desired replacement of *sopl*, which is shown schematically in Fig. 2A. A Southern blot of total DNA from one selected isolate, strain LSD3, and from the parent strain L33 is shown in Fig. 2B. The DNA was digested with *SacI* and hybridized to probes specific for *sopl* and *gyrB*. No *sopl* specific fragment was detected in LSD3 DNA confirming the loss of *sopl*. As shown in Fig. 2A, restriction of the introduced Nov^R cassette with *SacI* yields four fragments. In LSD3 DNA, the two large fragments (1.9 and 1.7 kb) gave strong hybridization signals with the *gyrB*-specific probe, while hybridization to the small internal fragments (<400 bp) became clearly visible only after prolonged exposure. The signal at about 2.2 kb which was seen in this restriction digest arose from cross-hybridization of the

Haloferax probe with *H. salinarium* DNA. This became evident by comparing the hybridization pattern of the LSD3 DNA with that of the parent strain L33 which showed two cross-hybridization signals at 1.7 and 2.2 kb. Of the two, only the hybridization at 2.2 kb fragment was visible in the DNA of LSD3, since the smaller fragment comigrated with the 1.7 kb fragment of the *Haloferax gyrB*.

The correct position of the Nov^R cassette relative to the *sopl* upstream sequences was demonstrated by the polymerase chain reaction (PCR) experiment shown in Fig. 2C. In genomic DNA of LSD3, the *gyrB* sequence could be amplified using a 5' oligonucleotide from the *sopl* upstream sequence and a 3' oligonucleotide complementary to the *gyrB* coding sequence, while no DNA product was obtained when an oligonucleotide from the *sopl* sequence was used as 3' primer. The location of the oligonucleotides used is shown in Fig. 2A.

In membranes prepared from the *sopl*-deleted strain LSD3, SRI-specific reactions could neither be detected in a Western blot using antibodies against the C-terminus of the protein (see Fig. 3) nor by monitoring the recovery of absorption at 590 nm over 1 s after actinic light illumination (Fig. 4). In the behavioural analysis shown in Fig. 5, LSD3 cells further lacked the photoresponses at 590 and 380 nm which are characteristic for the photoreceptor SRI. This confirmed that the fundamental phenotypic properties thus far attributed to SRI were eliminated by deletion of the *sopl* sequence.

Recovery of SRI synthesis and function via integrative transformation with a *sopl* expression plasmid

In an attempt to restore the synthesis of SRI in LSD3 cells

Fig.4. Spectroscopic analysis of cell-membrane preparations from strains expressing *sopl*. The light-induced absorbance difference spectra were recorded after 0.25 s of illumination. Membranes from P11 cells, containing the C-terminal truncated SRI, were twofold diluted with respect to membranes from M22 cells, expressing the *bop-sopl* hybrid gene and from the wild-type cells L33. The insets show the time-dependent recovery of absorbance at 590 nm after 2 s of actinic illumination.

Fig. 5. Behavioural analysis of the *sopI*⁻ deletion strain LSD3 and of LSD3 cells complemented with a *bop-sopI* expression cartridge. The distribution of reversals after orange light stepdown and u.v. pulse stimulation is shown. Each data point indicates the percentage of cells reversing after a period of 0.3 s. The filled symbols in the right column indicate the response to a u.v. pulse in the absence of orange background light. The time of stimulus application is marked by an arrow. The applied light stimuli were saturating.

and, additionally, to enhance the amount of protein produced, an expression plasmid was constructed in which *sopI* was placed under the control of the strong *bop* promoter. In the 1163 bp long expression cassette, which was synthesized by PCR, *sopI* was linked to the first 42 bp of *bop* and is further flanked by 400 bp of 5' and 200 bp of 3' non-coding sequences of the *bop* gene (Fig. 6B). The resulting hybrid gene encoded SRI fused to the BR presequence, which is presumed to support the efficient translation and insertion of BR into the membrane (Gropp *et al.*, 1992).

Aside from providing the regulatory sequences for efficient expression, the flanking *bop* sequences in the *bop-sopI* cartridge should induce homologous recombination of the hybrid gene with the copy of *bop* present on the genome, which has been inactivated by an insertional mutation (DasSarma *et al.*, 1983). The resulting exact replacement of the *bop* by the *sopI* coding sequence on the chromosome should represent the optimum genotype for high *sopI* expression. Targeting to the *bop* locus was

particularly favoured in the recipient LSD3, where potentially competing homologous sequences at the *sopI* locus are deleted. Using a similar gene-fusion approach, a further halobacterial retinal protein, the chloride pump halorhodopsin, could recently be expressed at a level comparable to BR (Heymann *et al.*, 1993).

For the transformation of halobacteria, the *bop-sopI* fusion cartridge was ligated into the *E. coli*-halobacterial shuttle vector pWL102ΔBA to give the *sopI* expression plasmid pEF198 (see the *Experimental procedures* and Fig. 6A). Except for the *sopI*-coding sequence this vector was identical to the *bop* expression vector pEF191 which allowed the expression of BR in *H. salinarium* at wild-type levels (Ferrando *et al.*, 1993). pEF191 has been shown to integrate as a single copy at the genomic *bop* locus. A Southern analysis of LSD3 cells transformed with pEF198 is shown in Fig. 7. Chromosomal DNA was digested with *Pst*I and, after transfer to a nylon membrane, hybridized with probes specific to *sopI* and *bop*. The *sopI*-specific fragment detected in the DNA of the isolate M22 was about 3.5 kb in size and not, as found in the plasmid, 2.7 kb. Thus, a rearrangement of the introduced plasmid had probably occurred. Hybridization with the *bop*-specific probe revealed that this rearrangement involved the chromosomal *bop* locus since the size of the labelled fragment differed from that of the untransformed recipient

Fig. 6. A. The *sopI* expression vector pEF198 used to restore SRI expression in the deletion strain LSD3. Its construction is described in the *Experimental procedures* and in Ferrando *et al.* (1993). *Mev*^R = mevinolin resistance; *Ap*^R = ampicillin resistance. *pHV2* is an endogenous halobacterial plasmid derived from *Haloferax volcanii* (Charlebois *et al.*, 1987).

B. Scheme of the *bop-sopI* expression cartridge of pEF198. **C.** The fusion between the BR and the SRI sequences. The black triangle indicates the processing site of the BR precursor peptide. The arrows show the direction of gene transcription.

Fig. 7. Genomic Southern analysis of LSD3 transformants. The same blot was hybridized subsequently to probes specific to *sopl* and *bop*. As a reference, an equimolar amount of isolated pEF198 DNA was analysed. All DNA were digested with *Pst*I.

strain (about 4.8 kb instead of 5.6 kb). The size of the *bop*- and *sopl*-specific *Pst*I fragments identified in the DNA of M22 was as predicted assuming homologous integration of pEF198 at the *bop* locus via a single crossover event involving the 400 bp long *bop* sequence 5' of *sopl*.

The recovery of the SRI phenotype in the recombinant strain M22 was investigated by Western analysis as well as by spectroscopic and motion analyses. The results are shown in the Figs 3, 4, 8 and 5, respectively. In cell-membrane preparations from this strain, a protein species was detected with a polyclonal antibody directed against the C-terminus of SRI (Fig. 3). This polypeptide displayed the same mobility, corresponding to 22 kDa, as SRI in membranes from the wild-type strain L33, suggesting that the BR precursor sequence is correctly processed in the expressed gene product. The higher-molecular-weight band observed in Western blots of M22 membranes is a dimer of SRI arising from incomplete dissociation. Aggregation to dimers and trimers is also observed in the wild type by increasing the amount of protein applied onto the gel (data not shown; see also Schegk and Oesterheld, 1988). Expression of the halorhodopsin-encoding gene fused to the same 5'-terminal sequence from the *bop*-coding sequence also yielded a correctly processed mature protein (Heymann *et al.*, 1993).

As judged from the absorption around 590 nm in the spectrum of M22 membranes (Fig. 8), the polypeptide translated from the *bop-sopl* expression cassette associated with retinal to form a chromophore-protein complex with the same absorption maximum as wild-type SRI. Upon illumination this pigment underwent a photocycle characterized by a decrease of absorption at 590 nm and the generation of a 380 nm intermediate as shown by the light-induced difference spectra in Fig. 4. The expressed pigment relaxed thermally to its initial state with a half-life of about 600 ms (see insets of Fig. 4). This value was

identical to that of wild-type SRI measured under the same conditions.

Transformation of the deletion strain LSD3 with pEF198 fully restored the wild-type phototactical behaviour. M22 cells responded to both a step decrease in attractant light intensity ($\lambda=563$ –600 nm) as well as to a blue light pulse under constant orange background illumination ($\lambda=366$ nm) (Fig. 5). This confirmed that both photochromic receptor systems ascribed to SRI (Spudich and Bogomolni, 1984) are indeed dependent on a single gene product and that both were functional and undistinguishable from the wild type in the reconstituted mutant.

The increase in SRI synthesis achieved by placing *sopl* under control of the *bop* promoter as estimated from the increase in absorbance at 590 nm (Fig. 8) and from the initial amplitude of light-induced absorbance decrease (insets to Fig. 4) was about four- to fivefold the wild-type expression level. This was still far below the desired increase of SRI to a BR-like level and indicated that the number of SRI copies per cell must be tightly controlled. Moreover, the target of the regulatory mechanism must reside either in the SRI protein or in the *sopl* coding sequence. This is, in fact, the only distinctive element of the *sopl* expression system compared to the high level *bop* expression system based on the vector pEF191.

A 3' truncated sopl enables high-level expression of the functional photoreceptor

A comparison of transcript levels (Fig. 9) in cells transformed with the *bop* expression vector pEF191 and M22 cells containing the *bop-sopl* expression cassette from pEF198 indicated a lower level of *sopl* mRNA than *bop* mRNA, although both genes had integrated at the same chromosomal locus and were under the control of the same regulatory sequences for transcription. A secondary structure analysis of *bop* and *sopl* mRNA (data

Fig. 8. Absolute spectra of membrane preparations of L33 cells and of LSD3 transformants expressing *sopl*. The samples were adjusted to the same total protein concentration. a, L33; b, M22; c, P11. Inset: difference spectrum of P11 and L33 membranes.

Fig. 9. Northern blot analysis of *sopl*-specific transcription in LSD3 transformants. Total RNA preparations were electrophoresed through a formaldehyde-agarose gel (A), blotted onto a membrane and then hybridized to a *sopl*-specific DNA fragment (B). The autoradiogram was developed after 40 min exposure. The endogenous *sopl* mRNA of L33 cells becomes visible only after two weeks of exposure (data not shown). In the lane labelled *bop* WT, RNA from strain L33 transformed with the *bop* expression vector pEF191 was analysed. This RNA was detected by using a labelled *bop* fragment employing the same amount of radioactivity as for the *sopl*-specific probe.

not shown) revealed a more extensive self-complementarity in the 3' region of *sopl* than in the corresponding stretch of *bop* sequence. To verify whether this region of the gene influences the stability and, thus, the steady-state level of *sopl* mRNA, the *sopl* coding sequence in pEF198 was deleted from base-pair 671 to base-pair 714 resulting in the modified plasmid pEF198 Δ C. This plasmid codes for an SRI mutant which terminates after Glu-223.

Transformation of LSD3 cells with pEF198 Δ C yielded transformants which had integrated the modified *bop-sopl* expression cartridge into the genomic DNA as already described for the plasmid pEF198. The Southern analysis of an individual isolate bearing the 3' deleted *sopl*, strain P11, is shown in Fig. 7. The amount of SRI produced in these cells, as estimated from the absorption spectrum of Fig. 8 (and inset) was about 6 nmol mg total protein⁻¹. This corresponded to a 10-fold increase over that in M22 cells and was approximately 40–50 times the level of natural expression. The resulting total yield of (C-terminally-truncated) SRI from P11 cells was about 2.5 mg l⁻¹ culture broth. A spectroscopic characterization of the overexpressed SRI is included in Fig. 4. The light-induced difference spectrum indicated that the truncation at the C-terminus neither affected the absorption behaviour of the two main intermediates, SR₃₇₃ and SR₅₈₇, nor influenced the photocycle kinetics of the pigment. The increase in the maximum amplitude of light-induced absorbance decrease (insets of Fig. 4) in P11 cells with respect to M22 cells matched the increase in absorbance observed in the absolute spectrum (Fig. 8), indicating that all of the expressed protein is photo-

chemically active. Significantly, the truncated photoreceptor restored the wild-type photoresponse in P11 cells (see Fig. 5). However, the behavioural assay employed here yields no information about the number of active photoreceptor molecules in these cells. Nevertheless, we can conclude from the observed physiological reaction that the C-terminal cytoplasmic portion of SRI is not essential for the release of phototactic signals. This provides a first step in the identification of those portions of the molecule which are needed to interact with the components of the signal transduction chain.

Surprisingly, Northern analysis of total mRNA from P11 cells (Fig. 9) revealed that the introduced deletion in the 3' end of *sopl* did not influence the steady-state level of the *sopl* specific transcript compared to M22 cells. The overexpression of SRI achieved with the truncated gene, therefore, must be exclusively due to an increase in translation efficiency or protein stability (see the Discussion).

Effect of the 3' end of *sopl* on *bop* expression

To determine if the 3' terminal sequence of *sopl* predictably alters the expression level of another retinal protein, we constructed a hybrid *bop* gene in which the 3' terminus has been replaced by the corresponding *sopl* sequence. In the resulting chimeric protein, Gly-220 of BR is fused to Tyr-210 of SRI. This fusion construct was cloned in pEF191 to give the vector pEF191SC. Transformation of the BR⁻ *H. salinarium* strain L33 with this plasmid yielded two different kind of transformants, A12/6 and A12/4. The first displayed the wild-type purple pigmentation resulting from BR synthesis; in the second weakly coloured transformant, the pigment level was clearly lowered. Genomic Southern blot and PCR analysis (data not shown; see Ferrando *et al.*, 1993) revealed that plasmid integration had restored the wild-type *bop* in the purple-coloured transformant A12/6, while in the pale clone A12/4, the introduced 3'-terminal hybrid gene was expressed. The steady-state level of the mRNA encoded by this gene was not significantly influenced with respect to the *bop* wild-type mRNA (data not shown). At the protein level, however, the replacement of the BR C-terminal sequence by that from SRI had a clear-cut effect, in which the level of the pigment was reduced by sixfold, as estimated from the absorption spectrum shown in Fig. 10. This result corroborated the data obtained with the truncated photoreceptor expressed in P11 cells.

Discussion

The study of the retinal proteins of *H. salinarium* as well as the investigation of its chemo- and photosensory system has thus far relied on the analysis of spontaneous or

Fig. 10. Absolute spectrum of membrane preparations from L33 cells synthesizing wild-type BR (A12/6) and a chimeric BR bearing the SRI C-terminus (A12/4). The samples contained the same amount of total protein.

randomly created mutant cells (Pfeifer *et al.*, 1984; Sundberg *et al.*, 1985; Soppa and Oesterhelt, 1989). One major drawback of this approach, however, is the possible occurrence of secondary, uncharacterized mutations which can contribute to the observed phenotype and may not be amenable to genetic complementation. We have already analysed two phenotypically SRI⁻ mutants in which photoresponses could not be reconstituted after transformation with the *sopl* expression plasmid pEF198 because of secondary genetic defects (Ferrando-May *et al.*, 1993). In this work we have described the construction of a genetically defined SRI-negative strain by the specific substitution of *sop I* with a novobiocin-resistance marker from *Haloferax* Aa 2.2. This strain is the first example of a specifically engineered deletion strain in an archaeal organism and represents a suitable recipient for future structure-function studies on SRI.

Targeting of the novobiocin cassette to the *sopl* locus occurred with high efficiency (55%). Nevertheless, transformants were obtained which had not recombined at the desired target site. The recombination site which is most likely to compete with *sopl* in this experiment is the endogenous DNA gyrase gene of *H. salinarium*. Its similarity to the mutated *gyr B* from *Haloferax* is consistent with the significant cross-hybridization obtained in the Southern analysis (Fig. 2). Considering the dimeric composition of DNA gyrase (Mizuuchi *et al.*, 1978) it must be further assumed, that the plasmid-encoded GyrB from *Haloferax* is able to functionally substitute for the *H. salinarium* GyrB subunit in the active heterodimeric enzyme complex.

At the phenotypical level, the deletion of the *sopl* sequence on the chromosome removed the spectroscopic and physiological features characteristic of the SRI protein, thus establishing the first direct experimental correlation between *sopl* and its gene product.

The upstream region of *sopl* harbours a further gene

involved in phototaxis, *htrl*, which codes for the putative methyl-accepting SRI signal transducer. *htrl* and *sopl* have been shown to be expressed as a single transcriptional unit (Ferrando-May *et al.*, 1993). In the *sopl*⁻ deletion strain, *htrl* has not been altered: the coding sequence stops at the original TGA, and transcriptional termination most probably occurs in the adjacent *gyrB* sequence. Nothing is known about the regulation of the *htrl-sopl* operon in *H. salinarium*. Our results indicate that the organization of the two genes into an operon is not essential for their correct translation to functional gene products, since expression of *sopl* from the *bop* locus in transformed LSD3 cells restores the wild-type physiological response of the cells. Moreover, the altered stoichiometric ratio of the two molecules in M22 and P11 cells does not dramatically influence the photoresponse. Only a slight reduction in the reaction to u.v. stimulation is observed in M22 and P11 cells compared to the wild type. This could be due to clonal differences, since *H. salinarium* populations show a distribution in response efficiencies which reflects the variability of the gain of the signal transduction chain. At present we cannot however exclude that either the expression level of the photoreceptor or its C-terminal truncation has an influence on the motility response. Further studies are in progress aiming at the quantification of the number of active photoreceptor molecules in these cells. This will allow determination of the influence of SRI overexpression on light sensitivity and will yield information about photoreceptor-transducer interaction (Krah *et al.*, in preparation).

As we have shown above, the overexpressed SRI is indistinguishable from the wild type by its photochemical and physiological properties, thus excluding a fundamental functional role of the C-terminus in the signal relay pathway. In this respect SRI differs from its eukaryotic counterpart, bovine rhodopsin, where the C-terminus plays an essential role in the modulation of the interaction between the receptor and the signal-transducing G-protein (Kühn, 1984).

A densitometric scan of the signal intensities in Northern blots of *sopl*-specific mRNA indicated that fusion of the *bop* promoter to *sopl* increases the steady-state level of the *sopl* transcript by at least two orders of magnitude compared to the wild type. This is only a rough approximation due to the differences in the time of exposure of the respective autoradiograms (see also Ferrando-May *et al.*, 1993). The stationary concentration of SRI, however, raises only by four- to fivefold, implying a control of the protein steady-state level. Additionally, the amount of *sopl*-specific mRNA obtained under the control of the *bop* promoter remained clearly below that of wild-type *bop* mRNA which indicated that there is also regulation at the mRNA level. Independent of the mechanism, a downregulating component must be located in the *sopl* coding

region. In fact, this is the only difference between the *sopl* transformants described in this work and cells expressing BR at wild-type levels after integration of the *bop* expression vector pEF191 into the genome (Ferrando *et al.*, 1993).

The deletion of 43 bp at the 3' terminus of *sopl*, which did not positively influence the *sopl* mRNA level, contributed to a 50-fold increase of SRI compared to the wild type. Moreover, the level of BR decreased in cells expressing a modified *bop* which carries the 3' terminal sequence of *sopl*. At present we cannot assess whether the enhanced stationary level of SRI in P11 cells is the result of an increase in translation of the *sopl* mRNA or of a reduced degradation rate of the protein. If the first hypothesis applies, then the 3'-terminal region identified would represent the binding site of a translational repressor such as those described for many bacterial and phage genes (McCarthy and Gualerzi, 1990). However, binding of the repressor to the target mRNA sequence, in most cases, induces rapid degradation of the mRNA (Petersen, 1992). This effect is not reverted by the deletion of this site in *sopl*. We currently favour the hypothesis that the copy number of SRI in *H. salinarum* is controlled through the modulation of the half-life of the protein and that its C-terminus possibly harbours a specific target for proteolysis. C-terminal truncations yielding higher level of expression have been observed also for two other membrane proteins, namely for the liver mitochondrial phosphate transporter expressed in *E. coli* (Ferreira and Pedersen, 1992) and for the turkey β -adrenergic receptor expressed in Sf9 insect cells (Parker and Ross, 1991). However, these are examples of heterologous expression systems, where problems arising from the incompatibility of the host translation and membrane insertion machinery with the expressed gene product are likely to be decisive. In the case of the expression of *sopl* in its homologous host, the influence of the C-terminus most likely reflects a true regulatory mechanism. Interestingly, a general correlation between the half-life of a protein and the half-life of its mRNA has been observed for inducible proteins, like SRI, which have to respond rapidly to environmental changes (Hargrove and Schmidt, 1989).

It is further intriguing to observe that the C-terminal sequence of SRI is enriched in prolines (P), glutamates (E), serines (S), and threonines (T) and is preceded by a cluster of basic residues. Local abundance of these amino acids flanked by basic residues has been proposed to constitute a signal for rapid protein destruction in Eucarya (Rogers *et al.*, 1986). According to a number of investigations, these so-called PEST sequences seem to have a more general structural significance rather than that of a sequence-specific degradation signal (Gottesman and Maurizi, 1992). The occurrence of PEST residues in the C-terminus of SRI, therefore, could

suggest that the structural requirements for the regulation of protein half-lives are conserved between Archaea and Eucarya.

As for the overexpression of the chloride pump halorhodopsin, the system established here for SRI production will greatly facilitate the investigation of the photoreceptor under several aspects. We are currently addressing questions as to the detailed assignment of photocycle intermediates and the state of the Schiff base in the SR₃₇₃ species (U. Haupts *et al.*, in preparation). Besides setting the stage for structural investigations, overexpression of SRI and SRI mutants will further bring significant support to the analysis of the interactions between SRI and the transducer molecule HTR1 and thereby contribute to a more general understanding of the molecular aspects of the halobacterial photosensing machinery.

Experimental procedures

Bacterial strains and transformation procedures

H. salinarum strain L33 (BR⁻, HR⁺, Rub⁻, Ret⁺) is a bacterio-opsin-deficient mutant of S9 (Wagner *et al.*, 1981). Halobacteria were grown in peptone medium (Oesterhelt and Stoebenius, 1973). The transformation method of Cline *et al.* (1989) modified as described in Ferrando *et al.* (1993) was used. Mevinolin was added to the growth medium at a concentration of 25 μ M. Novobiocin was used at a concentration of 2 μ g ml⁻¹ in liquid medium and at 1 μ g ml⁻¹ for agar plates. *Escherichia coli* strain XL1-Blue was obtained from Stratagene, strains MV1190 and CJ236 were from Bio-Rad. *E. coli* strains were grown in LB or minimal medium containing the appropriate antibiotic and were transformed according to either the CaCl₂ or the RbCl procedure.

Oligonucleotides

The oligonucleotides used for site-directed mutagenesis and sequencing were synthesized on an automatic DNA synthesizer (Applied Biosystems, 381 A).

The following oligonucleotides were employed for site-directed mutagenesis: EcoRV5': 5'-GGGTGGTGCGTGATAT-CCGCCGTCGCAACC-3'; 3' SOP: 5'-TTAGCGGCCGCTCA-GTCCGCCGCCGTCGCCTC-3'; 5' BOP: 5'-GTGCGGATCCG-ACGTGAAGATGGGGCTCCC-3'; LINKING: 5'-GCGGTTGC-GACGGCGTCCATCTGCGATACCCCTCC-3'; SOP Δ C: 5'-CGTGTTTCATGCACTCGGAGTGACTGAGCGGCCGCGACC-AG-3'; BOPCSOP: 5'-CGCCGCGCGTAGAAGAAATACCCG-AAGCCGACCTTCGCGCTCA-3'.

Plasmid constructions

If not otherwise indicated, recombinant DNA techniques were performed according to Sambrook *et al.* (1989).

sopl-deletion plasmid pBB

Construction (Fig. 1) started from the plasmid pSopFS, which

carries a 5.8 kb fragment of *H. salinarium* L33 chromosomal DNA subcloned from the genomic isolate A2H11 (Blanck *et al.*, 1989). This *Bam*HI–*Eco*RI fragment carries *sopI* together with 2.9 kb of genomic sequence upstream and 2.1 kb downstream of the gene. A unique *Eco*RV restriction site is located 106 bp 3' to the *sopI* stop codon. A second *Eco*RV site was introduced at the start codon of *sopI* using the oligonucleotide *Eco*RV5' and following the procedure of Kunkel *et al.* (1987). From the resulting plasmid pSopFS–*Eco* the *sopI* sequence was removed by digestion with *Eco*RV and the remaining vector fragment was ligated to a blunt-ended DNA fragment from the plasmid pMDS1 (Holmes *et al.*, 1991) carrying the novobiocin-resistant *gyrB* gene from the halobacterial genus *Haloferax* Aa 2.2 (phenon K). In the final construct pBB, *sopI* plus 106 nucleotides of the 3' flanking region had been replaced by the resistance marker. The upstream sequence bearing the *htrI* gene was left intact.

sopI-expression plasmids pEF198 and pEF198ΔC

Construction of the *bop*–*sopI* expression cartridge shown in Fig. 6B was performed by PCR following a protocol described by Yon and Fried (1989) with some modifications. In a first step, a *bop*-specific fragment was synthesized carrying at its 3' end the first 20 bp of the *sopI*-coding sequence. This fragment was amplified from the *bop*-plasmid pHVΔB13 (H. Vogelsang and D. Oesterhelt, unpublished data) using the oligonucleotide primer 5'BOP which hybridizes at the *Bam*HI site 389 nucleotides upstream of the *bop* ATG and the LINKING oligonucleotide. The resulting PCR product contained the *bop* promoter region, the first 42 bp of *bop* encoding the BR presequence, and the first 20 bp of *sopI*. In the secondary PCR the purified *bop* promoter fragment with the 3' *sopI*-specific overlap was combined with a DNA fragment carrying the entire *sopI* gene. The resulting heteroduplex DNA product was amplified with the oligonucleotides 5'BOP and 3'SOP. The primer 3'SOP introduced a new *NotI* site after the *sopI* ATG. Both PCRs were performed using standard conditions as described by the manufacturer of the Taq polymerase (Amersham). The obtained fusion product was cloned in a *Bam*HI–*NotI*-digested pEF1290 (Ferrando *et al.*, 1993) which provided the putative *bop* terminator. The sequence of the hybrid *bop*–*sopI* gene in the resulting plasmid pEF1298 was verified by DNA sequence analysis according to the USB Sequenase protocol. Finally, the *Eco*RI–*Hind*III fragment from pEF1298 which encompassed the *Bam*HI–*NotI*-delimited expression cartridge was ligated into *Eco*RI–*Hind*III digested pWL102ΔBA to yield the plasmid pEF198 (see also Ferrando *et al.*, 1993).

For the deletion of the C-terminus of SRI, 44 bp in the 3' coding region of *sopI* were removed by oligonucleotide-directed mutagenesis using the oligonucleotide SOPΔC. The modified expression vector pEF198ΔC was obtained as already described for the plasmid pEF198.

Vector pEF191SC for the expression of a 3' *bop*–*sopI* hybrid

The replacement of the *bop* coding sequence from base pairs 1093–1161 with the last 93 bp of *sopI* coding sequence was carried out in a manner similar to that described in the preceding paragraph. A *bop* fragment carrying a 3' extension

encompassing the fusion point was synthesized by PCR using the oligonucleotides 5'BOP and BOPCSOP. This primary product was combined in a second PCR with *sopI* DNA. In the resulting PCR product the *bop* and *sopI* sequences were joined at the desired position, as confirmed by DNA sequencing. The PCR product was then cloned in *Bam*HI–*NotI* digested pEF1290 and subsequently transferred to the halobacteria–*E. coli* shuttle vector pWL102ΔBA to give the final construct pEF191SC. This plasmid encodes a BR–SRI hybrid, in which the last 28 amino acids of the mature BR are missing and have been replaced by 29 amino acids from the SRI sequence.

Southern and Northern analysis

Isolation of total halobacterial DNA and RNA was performed as previously described (Ferrando *et al.*, 1993). For Southern blot analysis, 2 μg of total halobacterial DNA were digested with either the restriction endonuclease *SacI* or *PstI*, separated on a 1% agarose gel and transferred to a Hybond N membrane (Amersham) by vacuum blotting. Hybridization probes were synthesized by incorporation of digoxigenin-labelled dUTP (Boehringer-Mannheim) in *bop*- or *sopI*-specific DNA fragments via PCR. The reaction mixtures contained 50–100 ng DNA, 40 μM dNTP each (Pharmacia), 20 μM DIG-11-dUTP, 1 μM of each primer, and 2.5 U Taq polymerase in 1× reaction buffer (Amersham). The following cycling conditions were applied: denaturation (94°C, 1 min), annealing (53°C, 1 min), extension (72°C, 1 min), 30 cycles. The filters were prehybridized for 1 h at room temperature in hybridization buffer (50% (v/v) formamide, 5× SSC, 2% (w/v) blocking reagent, 0.1% (w/v) *N*-laurylsarcosine, and 1% (w/v) SDS). For hybridization, filters were incubated overnight at 42°C in hybridization buffer with 10 μl of the heat-denatured PCR probe. The membranes were then submitted to the following washing steps: 2× 15 min in 2× SSC, 0.1% SDS at room temperature; 2× 15 min in 0.1× SSC, 0.1% SDS at 68°C. The chemiluminescent detection of the labelled fragments was performed as described in the instructions to the DIG Luminescence Detection Kit supplied by Boehringer-Mannheim.

Isolated halobacterial RNA was subjected to DNase treatment as described in Tullis and Rubin (1980). Northern analysis with radioactively labelled *sopI*- and *bop*-specific probes has been described previously (Ferrando *et al.*, 1993).

Isolation of SRI-containing membranes.

For protein isolation, transformants expressing *sopI* were grown under mevinolin selection in 700 ml cultures until late stationary phase. The cells were then harvested, resuspended in 10 ml of buffered basal salts (4.3 M NaCl, 81 mM MgSO₄, 26 mM KCl, and 10 mM MES, pH 6.0) containing 20 μg ml⁻¹ DNase I and 0.03% (w/v) phenylmethylsulphonyl fluoride and dialysed overnight against distilled water at 4°C. Total membranes were collected by centrifugation at 110 000 × *g* at 10°C for 1.5 h and then washed twice in 2–3 ml of low-salt buffer (0.1 M KCl, 10 mM MgCl₂, 10 mM MES, pH 6.0). Samples were adjusted to the same protein concentration and submitted to spectroscopic or Western analyses.

Preparation of a polyclonal anti-SRI immunoserum and Western analysis

Antibodies directed against a C-terminal peptide of SRI were raised in rabbits. As an antigen, a peptide corresponding to amino acid residues 222–238 was synthesized by Merrifield solid-phase synthesis on a semi-automatic Labotec peptide synthesizer SP640. The peptide was then coupled to keyhole limpet hemocyanin according to Doolittle (1986). Rabbits were immunized with 250 µg of carrier-bound peptide at intervals of three and two weeks. Blood was taken 10 d after the last injection.

For gel electrophoresis, samples containing 20 µg of total membrane protein were solubilized in DB buffer (100 mM Na₂CO₃, 100 mM DTT, 4% SDS, 24% sucrose) by heating for 1 h at 43°C with short incubations in an ultrasonic bath. Electrophoresis was performed according to Schaeffer and Jagow (1987) in a Pharmacia Midjet Electrophoresis System. The separated proteins were then transferred to a nitrocellulose membrane (Schleicher and Schuell) using a Sartorius Semi-Dry Blotting apparatus. Detection of SRI occurred with a donkey-antirabbit horseradish peroxidase-linked secondary antibody in conjunction with the ECL Western blotting detection system (Amersham).

Spectroscopic analysis

Light-induced absorption difference spectra of membranes containing SRI were recorded in a laser diode-array spectrophotometer as described in Uhl *et al.* (1985). The light-induced recovery of the SRI initial state was measured at 590 nm in an Aminco DW2000 spectrophotometer. The samples were illuminated for 2 s with actinic light filtered through a cut-off filter (OG 570, Schott).

Motion analysis

Cells were grown for 2.5 days, the culture diluted with nutrient broth to a density of $0.5 \times 10^9 \text{ ml}^{-1}$ and supplemented with 0.1% arginine as fermentative energy source (Hartmann *et al.*, 1980). After incubation for at least 30 min in the dark, the measurements were performed at 22°C with infra-red observation light. Orange stimulus light was produced by a 900 W Xe lamp and selected by 2 cm of a 2% (w/v) CuCl₂ solution, an OG 570 (Schott), both immersed in a water bath (20 cm in length) and a broad-band interference filter (570–643 nm). Ultraviolet light was generated by a 100 W mercury lamp and the 366 nm line was selected by a WG 360 cut-off combined to a UG1 band pass filter (Schott).

The cells were exposed to orange light with an intensity of 303 W m^{-2} . In one-minute intervals the light was switched off for a period of 10 s (orange off stimulation). Alternatively, a pulse of 366 nm light (560 W m^{-2}) was applied on a constant orange background. The pulse duration was 5 s. To avoid excitation of SRII, the u.v. light was filtered through a 50% NG filter (Schott). The low-intensity pulse did not elicit any response in the absence of orange background illumination (see filled symbols in Fig. 5). The response of the cells was recorded by a computerized cell-tracking system (Motion Analysis) with a frame rate of 10 s^{-1} . The experimental setup and the motion

analysis algorithm are described elsewhere (Marwan and Oesterhelt, 1990).

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