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Genetic transfer of the pigment bacteriorhodopsin into the eukaryote *Schizosaccharomyces pombe*

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The gene encoding for bacterio-opsin (*bop* gene) from *Halobacterium halobium* has been introduced in a yeast expression vector. After transformation in *Schizosaccharomyces pombe*, bacterio-opsin (BO) is expressed and was detected by antisera. The precursor protein of BO (pre-BO) is processed by cleavage of amino acids at the N-terminal end as in *H. halobium*. Addition of the chromophore, retinal, to the culture medium results in a slight purple colour of the yeast cells indicating the in vivo regeneration of BO to bacteriorhodopsin (BR) and its incorporation into membranes. Therefore, in contrast to the expression in *E. coli*, isolation of the membrane protein and reconstitution in lipid vesicles is not necessary for functional analysis. The kinetics of the ground state signal of the photocycle BR in protoplasts is demonstrated by flash spectroscopy and is comparable to that of the natural system. The present investigation shows for the first time the transfer of an energy converting protein from archaebacteria to eukaryotes by genetic techniques. This is a basis for further studies on membrane biogenesis, genetics, and bioenergetics by analysis of in vivo active mutants.

Bacteriorhodopsin; Fission yeast; Gene expression; Eukaryote; Protein regeneration; (*Halobacterium halobium*)

1. INTRODUCTION

Bacteriorhodopsin, the only protein of the purple membrane (PM) of *Halobacterium halobium*, translocates protons from the inside to the outside of the cell [1]. The gene of bacterio-opsin (*bop* gene) has been analysed [2,3], but a homologous transformation system with plasmids containing selection markers has not been developed for the extreme halophile *H. halobium*. The *bop* gene encodes a precursor protein (pre-BO) of 262 amino acids. Pre-BO has, in addition to the 248 amino acids of the mature protein, a 13-amino acid prolonged N-terminus and an extra asparagine at the C-terminus. No experimental data are available on how the seven transmembrane helices of BR enter the membrane and whether the short presequence is involved in the membrane insertion mechanism. No approach has been published about the expression of the authentic *bop* gene with its own codon usage. The present investigation shows the successful expression and in vivo regeneration of the pigment in the fission yeast *Schizosaccharomyces pombe*. Several advantages of this approach to earlier expression studies with modified bacterio-opsins in *Escherichia coli* are demonstrated.

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Abbreviations: BR, bacteriorhodopsin; BO, bacterio-opsin; PM, purple membrane; pre-BO, precursor protein of bacterio-opsin

2. MATERIALS AND METHODS

2.1. *Cloning procedure*

A 5.1 kb *Pst*I restriction fragment containing the *bop* gene can be isolated from *H. halobium* and subcloned in the plasmid pBR322 [2,3]. A 1.6 kb *Bam*HI/*Bst*EII fragment from the resulting plasmid was subcloned in pUC8. For further construction we used the 1.6 kb *Bam*HI/*Hin*dIII fragment (the *Hin*dIII site derives from the polylinker), which contains the sequence

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of the structural gene (786 bp) and about 400 nucleotides of noncoding sequences at both ends (fig.1). These noncoding sequences were removed together with the 5′- and 3′-terminus of the gene by *Aat*II and *Not*I digestion. The shortened gene fragment was first ligated with synthetic duplex DNA containing *Not*I and *Hin*dIII protruding ends to restore the last 19 bp of the *bop* gene including the stop codon. Subsequently the fragment was ligated with duplex DNA containing *Bam*HI and *Aat*II protruding ends. This duplex restores the first 60 bp of the natural gene. The gene was sequenced in pBSM13+ to confirm that no modification in the coding region was introduced and cloned into the polylinker of the yeast expression vector pEVP11. In the resulting plasmid pEVBOp (fig.2) the gene is located downstream of the constitutive alcohol dehydrogenase promoter of *S. pombe*. The vector pEVP11 and the plasmid pEVBOp were used for transformation [4] of *S. pombe* strain leu 1-32 h^-. Intact yeast cells are transformed with 1 μg DNA in the presence of 40% PEG 4000 and alkali cations (300 mM lithium acetate). Yeast transformants were selected by growth on EMM2 minimal medium [5] agar plates without leucine.

2.2. *Isolation of total cellular protein*

Transformed *S. pombe* cells were grown two days to the stationary phase in EMM2 minimal medium with shaking at 30°C. The cells were collected by centrifugation and broken by vortexing with glass beads. Protein extracts were boiled in sample buffer [6] for 2 min and loaded onto a 12.5% (w/v) SDS-polyacrylamide gel. After electrophoresis the protein was transferred to nitrocellulose filter. The blot was incubated with rabbit antisera. Antisera were raised by injection of 5 μg native purple membranes/animal. Antibody binding was detected by horseradish peroxidase-IgG and subsequent enzymatic reaction [7].

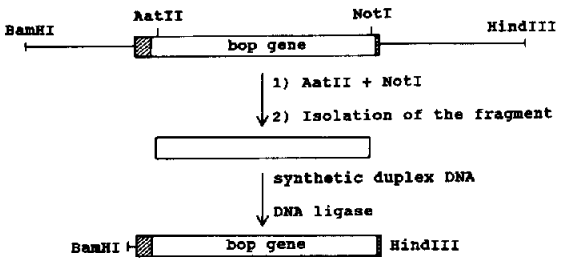

Fig.1. Schematic representation of the *bop* gene encoding for the bacterio-opsin precursor protein. Oligonucleotides were synthesized at the Freie Universität Berlin, Institut für Biochemie, by Dr R. Bald.

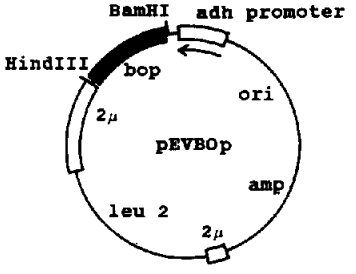

Fig.2. Plasmid map of pEVBOp. The expression vector for *S. pombe* carries the *bop* gene (black box) downstream of the alcohol dehydrogenase promoter from *S. pombe* [13]. The selection marker for *S. pombe* transformants is the *leu* 2^+ gene.

2.3. *Flash spectroscopy*

The BR photocycle (5°C, pH 7.5) was excited by a short (5 ns) laser flash (580 ± 10 nm, 1 mJ) and subsequent absorbance changes in the samples were monitored in 90° geometry (optical pathlength 1 cm). Signals depicted are the average of 200 single flashes (repetition rate 0.1 Hz).

3. RESULTS AND DISCUSSION

3.1. *Western blot revealed processing of the precursor protein*

The expression of BO derived from the *bop* gene downstream of a yeast promoter was controlled by Western blot analysis (fig.3). Lane 1 shows the pattern of purple membranes from *H. halobium*, which was interpreted previously as incomplete processing of the precursor protein [7]. It was shown that the upper band is the precursor protein and the middle band is a slightly shortened precursor, where part of the presequence is cleaved off. The lower band is the mature protein. Interestingly there are only two protein bands visible for the pEVBOp extract (lane 3), the lower one comigrates with the mature protein (lane 1, lower band) and the higher one correlates to the shortened precursor protein (middle band). This pattern

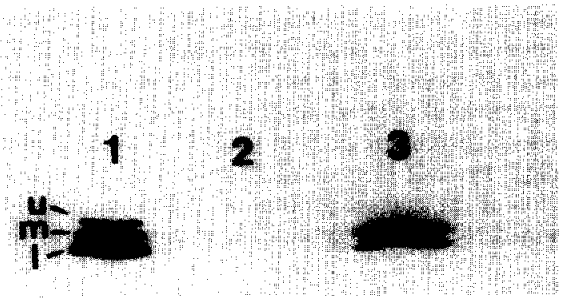

Fig.3. Western blot analysis of proteins synthesized in *S. pombe*. Lane 1, control: 350 ng of purple membrane from *H. halobium* strain ET 1001 isolated as described earlier [14]. Lanes: 2, 200 μg total protein extract of cells transformed by the vector pEVP11; 3, 200 μg total protein extract of cells transformed by pEVBOp containing the *bop* gene. U, upper protein band; M, middle protein band; L, lower protein band.

indicates that the precursor is processed to the mature protein. In contrast to *E. coli*, the constitutive expression of BO in *S. pombe* cells is possible without degradation. In addition the pEVBOp transformants do not reveal any reduction of growth compared to pEVP11 transformants. Therefore natural BO expressed in *S. pombe* overcomes the problems inherent to the expression of BO fusion proteins in *E. coli* [8,9].

3.2. *In vivo regeneration of the pigment*

Two important questions should be answered: Does BO fold into *S. pombe* membranes in the same way as in *H. halobium* and does the protein display the same functional characteristics? A sensitive feature for the native conformation are the spectroscopic properties of its chromophore (retinal) which was added to the cultures to a final concentration of 2 μM. The cells transformed with pEVP11 had a milky, colourless appearance whereas the pEVBOp transformants showed a slight purple colour. Differential absorbance spectroscopy of cells with retinal against those without retinal (fig.4) showed an absorbance peak at 568 nm, as was found for purple membranes [1]. This is the first indication that in vivo regeneration of heterologously expressed BO by retinal was successfully performed. In vivo regeneration of the pigment is also known for *H. halobium* mutants (JW5) [10] which are not able to synthesize the chromophore but is not reported for BO expressed in *E. coli*. BO expressed in *E. coli* has to be isolated and reconstituted in the lipid vesicles to show functional properties [8,9].

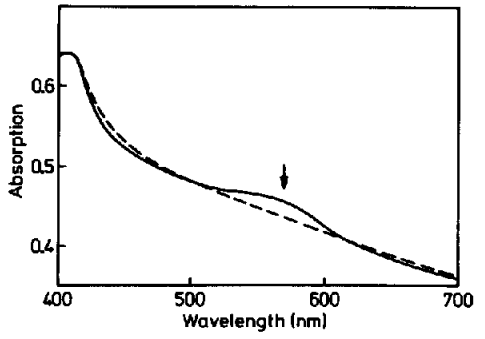

Fig.4. Difference absorption curve of transformed yeast cells. Continuous line: pEVBOp transformant cells with retinal against those without retinal. Broken line: pEVP11 transformant cells with retinal against those without retinal.

3.3. *Recovery of the ground state of the photocycle*

For analysing the photocycle of BR protoplasts of *S. pombe* [11] were used. Under in vivo conditions, time-dependent absorbance changes during the photocycle of BR at 550 nm have been measured. Fig.3a shows the recovery of the bleached ground state BR-568 after flash excitation of the photocycle. The observed kinetics is similar to data obtained from native PM [12].

3.4. *Conclusion*

Neither a homologous nor a heterologous transformation system for the *bop* gene leading to photoactive pigments in vivo has been reported. This eukaryotic expression system displays a threefold advantage: (i) the constitutive expression of an archaebacterial gene in yeast is possible without modification of the coding region; (ii) the integral membrane protein BR is accumulated in cellular membranes where it is protected from proteolysis. A fusion with leader sequences of yeast proteins is unnecessary. (iii) In vivo regeneration of the pigment is possible by addition of its chromophore.

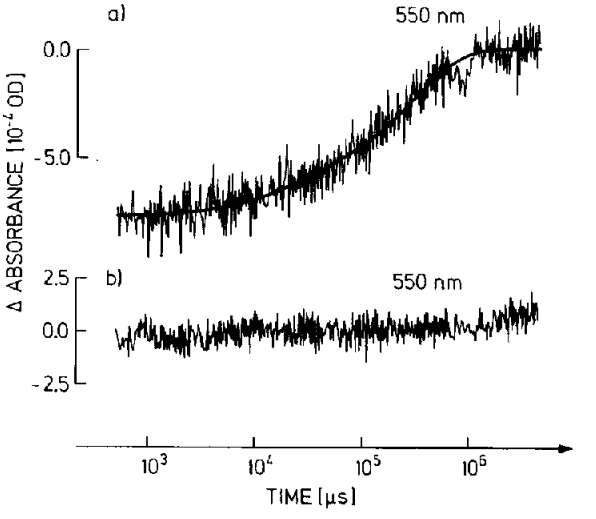

Fig.5. Flash-induced absorbance changes of BR in *S. pombe* transformants at 5°C. (a) The time-dependent absorbance change at 550 nm for protoplasts of pEVBOp transformants shows the reappearance of the BR-568 photocycle ground state, which was bleached by a laser flash. This bleaching is not resolved in time. (b) In a control experiment no flash-induced absorbance changes were detected at 550 nm for protoplasts of pEVP11 transformants.

Isolation of the protein and reconstitution in lipid vesicles is not necessary for functional analysis as for the *E. coli* system [8,9]. For further genetic studies and functional analysis *S. pombe* might be an appropriate expression system for halobacterial retinal proteins. This enables the investigation of directed protein transport and membrane insertion as well as bioenergetical studies.

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