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Homologous bacterio-opsin-encoding gene expression via site-specific vector integration

(*Halobacterium halobium*; archaebacteria; shuttle-mutagenesis vector; transformation; IS*H* element; genomic integration; *bat*; *brp*; *bop*)

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SUMMARY

Homologous recombination in the archaebacterium *Halobacterium halobium* has been investigated and exploited for the wild-type (wt) level of expression of the bacterio-opsin-encoding gene (*bop*). The *Haloferax volcanii-Escherichia coli* shuttle vector, pWL102, was used to construct a shuttle-mutagenesis vector, pEF191, bearing *bop* and short flanking sequences. Transformation of a bacteriorhodopsin (BR)-negative *H. halobium* strain with pEF191 resulted in plasmid integration at the homologous *bop* locus. A model for this site-specific vector integration is presented which has been confirmed by determining the arrangement of the repeated homologous sequences on the chromosome. Two different configurations are obtained after integrative transformation due to the presence of an insertion element in the genomic copy of *bop*. In one configuration, the functional *bop* cluster containing the regulatory *bat* and *brp* genes was in wt arrangement. In the second configuration, the *bop* cluster is interrupted by 10 kb of plasmid vector sequences, and the upstream region required for *bop* expression was limited to 400 bp. The BR production for both configurations was determined and found to be at wt level. These results suggest that the function of the putative *bop* promoter does not depend on the defined upstream positions of *bat* and *brp*. The system presented here can be easily exploited for structure-function studies on BR and introduces homologous gene targeting as a powerful tool in the study of halobacterial genetics.

INTRODUCTION

The seven-helix motif is a structural feature characteristic of the family of eukaryotic rhodopsins. In the prokaryotic world, this motif occurs only in the branch of archaebacteria. The most abundant and well-studied member of the archaebacterial seven-helix protein family is BR which in the archaebacterium *H. halobium* enables photophosphorylation by light-driven proton pumping (Oesterhelt and Stoeckenius, 1973). In virtue of its thermo- and photochemical properties, BR provides a biological material of great interest for technical applications (Oesterhelt et al., 1991) and represents also a conserved structural framework which may be favourable for the engineering of membrane structures with variable functions.

The structure-function studies performed so far on BR have relied on either the generation and selection of modified BRs after in vivo random mutagenesis (Soppa and Oesterhelt, 1989) or the expression of site-directed mu-

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Abbreviations: Ap, ampicillin; *bat*, gene encoding the putative BR-activating protein; *bop*, gene encoding bacterio-opsin; bp, base pair(s); BR, bacteriorhodopsin; *brp*, gene encoding the putative BR-related protein; cpm, counts per minute; Δ, deletion; EtdBr, ethidium bromide; *H.*, *Halobacterium*; *Hf.*, *Haloferax*; HMGCoA, 3-hydroxy-3-methylglutaryl coenzyme A; IS*H*, halobacterial insertion element; kb, kilobase(s) or 1000 bp; L33-pE191, *H. halobium* strain L33 with plasmid pE191 integrated into its genome (see section b and Fig. 1); Mev, mevinolin; nm, nanometer; nt, nucleotide(s); *ori*, origin of DNA replication, p, plasmid; PCR, polymerase chain reaction; PEG, polyethylene glycol; ^{R}, resistance/resistant; REG, regeneration; SDS, sodium dodecyl sulfate; wt, wild type.

tants in *E. coli* (Khorana, 1988). The transformation system for halobacteria developed by Cline et al. (1989) permits the reintroduction and expression of *bop* in its homologous host, as reported by Ni et al. (1990). The BR negative phenotype of the *H. halobium* strains available as recipients for *bop* expression is a result of the inactivation of the chromosomal copy of *bop* by an insertion element (DasSarma et al., 1983). These strains therefore still carry the entire endogenous *bop* sequence, albeit interrupted, with the potential to undergo homologous recombination with the copy of *bop* borne on a plasmid. A reliable *bop* expression system in these strains may be based either on a stable self-replicating *bop* expression vector or on a specific recombination event of the cloned *bop* sequences into the genome. A *bop* expression vector, which was derived from a plasmid isolated from *Halobacterium* sp. GRB, failed to produce wt amounts of BR due to plasmid instability (Krebs et al., 1991).

In this work we attempted to develop a homologous *bop* expression system at wt level by means of site-specific vector integration. Homologous recombination has been successfully exploited in several organisms as a tool for the analysis of gene expression (Vasseghi and Claverys, 1983; Orr-Weaver et al., 1983; Capecchi, 1989; Gal et al., 1991). The aim of the present study was to document the occurrence of homologous recombination between newly added exogenous DNA and chromosomal sequences in an archaebacterial strain and to propose a model which elucidates the mechanism of vector integration into the halobacterial genome. Furthermore, the exact mapping of the recombination locus yields information concerning the influence of upstream regions on the expression of *bop*. Finally, the system we studied should be suitable for the effective production and expression of *bop* mutants.

RESULTS AND DISCUSSION

(a) Construction of an *Escherichia coli-Halobacterium halobium* shuttle expression vector

For the development of a *bop* expression system for *H. halobium* we utilized the *Hf. volcanii-E. coli* shuttle vector pWL102ΔBA which is a derivative plasmid of pWL102 (Lam and Doolittle, 1989). This plasmid has an *ori* derived from *Hf. volcanii* and is stable in the restriction minus *H. halobium* strain PO_3 after transformation (Blaseio and Pfeifer, 1990).

The construction of the *bop* expression vector pEF191 is outlined in Fig. 1. It is a phasmid vector which contains both an *E. coli* and a halobacterial *ori* together with the Ap^R and Mev^R markers and therefore may be propagated and selected for in both organisms. The presence of the intergenic region from the phage f1 allows for the isolation of pEF191 as single-stranded DNA, enabling site-directed mutagenesis as well as sequencing without any further cloning steps.

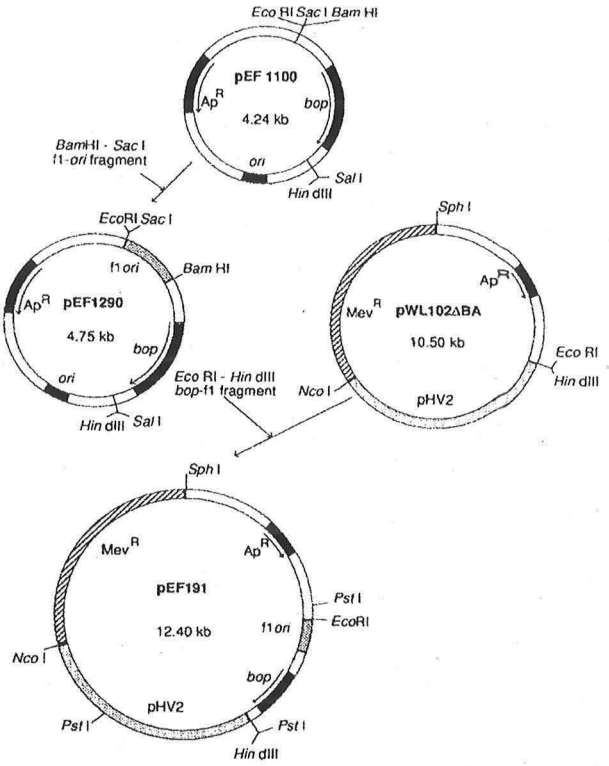


Fig. 1. Construction of the *bop* expression and mutagenesis vector pEF191. The starting plasmid pEF1100 carries a 1.4-kb genomic fragment which comprises *bop* and 388 bp upstream from the start codon and 200 bp downstream from the stop codon. The 0.5-kb *Bam*HI-*Sac*I fragment bearing the f1 *ori* was isolated from the plasmid pUCf1 (Pharmacia). This fragment was cloned into pEF1100 DNA, resulting in the vector pEF1290. The final construct, pEF191, was obtained by excising the 1.9-kb *bop*-f1 fragment from pEF1290 with *Eco*RI+*Hin*dIII and ligating it into pWL102ΔBA DNA which had been digested with the same enzymes. pWL102ΔBA is a derivative plasmid of the *E.coli-Hf.volcanii* shuttle vector pWL102 (Lam and Doolittle, 1989), which is missing the *Hin*dIII site between the halobacterial *ori* and the Mev^R. The pWL102ΔBA plasmid DNA was a kind gift of M. Mevarech. **Methods:** Transformation of *H. halobium* with plasmid DNA was performed following the procedure described by Cline et al. (1989) with some minor modifications: after treatment with PEG600, spheroplasts were diluted with 9.5 ml REG solution (3.5 M NaCl/150 mM $MgSO_4 \cdot 7H_2O$/50 mM KCl/7 mM $CaCl_2$/50 mM Tris·HCl pH 7.2/15% sucrose), pelleted and resuspended in 1 ml *H. halobium* complex liquid medium as described (Cline et al., 1989). After 12 h of gentle shaking at 37° C, aliquots of 50–100 µl were spread on complex support medium containing 25 µM Mev.

The *Bam*HI-*Sal* I genomic DNA fragment from *H. halobium* contained in pEF191 comprises *bop* and short flanking sequences, 388 bp upstream from the start codon and 200 bp downstream from the stop codon. In the non-coding regions, putative control elements for transcription and translation have been localized by comparison

with halobacterial consensus sequences (Brown et al., 1989).

(b) Transformation of *Halobacterium halobium* with the *bop*-expression vector pEF191

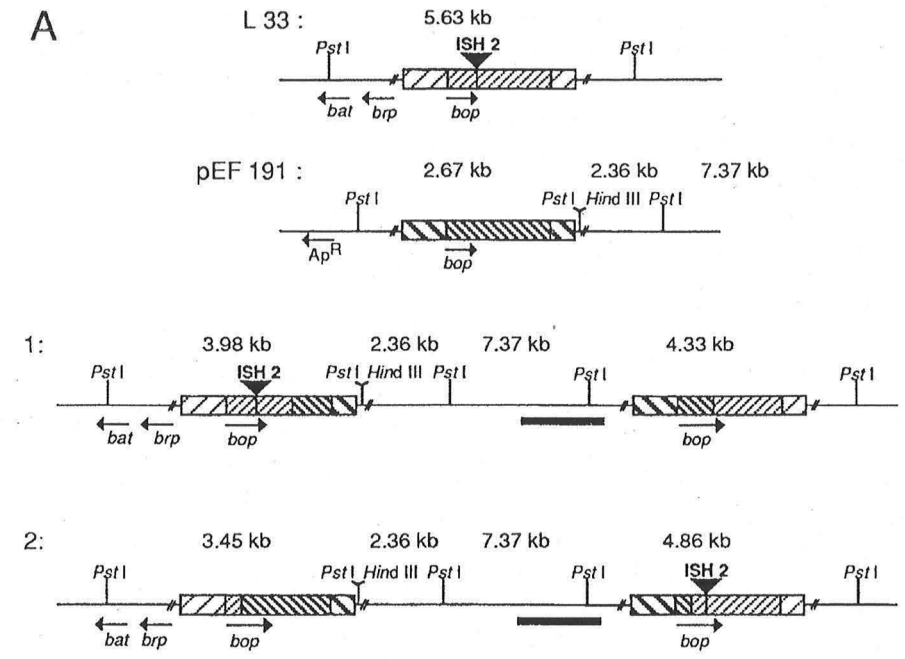
In the BR-deficient *H. halobium* strain L33 the chromosomal copy of *bop* is inactivated by the stable insertion of the 520-bp long IS*H2* element (DasSarma et al., 1983). When L33 was transformed with pEF191 DNA isolated from *E. coli*, 10^3 Mev^R transformants per μg of DNA were obtained (details of the transformation procedure are described in the legend to Fig. 1). Due to BR production 50–70% of these colonies were purple in colour. The high background of BR-negative transformants which were able to grow on Mev-containing medium may be due to a partial incorporation of the plasmid DNA with the concomitant rescue of the Mev^R gene. This has been described previously for the transformation of a restriction-positive *Hf. volcanii* strain with *E. coli*-derived pWL102 DNA (Blaseio and Pfeifer, 1990). The spontaneous occurrence of Mev^R halobacteria was comparatively low, 1–5% as judged by the outcome of mock transformations.

Colony hybridization revealed that >90% of the Mev^R L33 transformants, which expressed *bop*, had also retained the *E. coli* portion of the vector. We analysed four of these clones by Southern analysis and found that none of them contained the plasmid in its self-replicating form. In three of the analysed transformants, the plasmid DNA had been inserted into the genome at the homologous *bop* locus. In the fourth isolate, integration had occurred at a nonhomologous site and therefore was not characterized further. Integrative transformation in *H. halobium* has also been detected with other pEF191-derived plasmids carrying mutant *bop* as well as the sensory rhodopsin I-encoding gene and hybrid halobacterial genes (E. F. and D. O., manuscript in preparation). No transformants expressing these genes were found in which the plasmid was present as an extrachromosomal element. Integration into the genome may be induced by linear DNA molecules which could arise by restriction of the *E. coli*-derived plasmid DNA in L33. This may be due to a restriction function present in L33 cells but absent in the restriction-minus *H. halobium* strain in which stable replication of pWL102 has been demonstrated (Blaseio and Pfeifer, 1990).

Fig. 2A illustrates the proposed mechanism of homologous recombination occurring between the plasmid and the chromosomal *bop*. According to this model integration of the plasmid is the result of a single cross-over event, which may occur on either side of the IS*H2*. This construction results in a duplication of the identical sequences and restores one wt copy of the gene. This recombination mechanism has been confirmed by Southern analysis. Fig. 2B and C show the result of the analysis of two L33-pEF191 transformants, L33-pEF191/1 and L33-pEF191/2, which had integrated the plasmid on different sides of the insertion element. In the third isolate identified, which is not shown in the figure, the side of integration was identical to that of L33-pEF191/2. Total DNA isolated from L33 pEF191/1 and -/2 was cut with *Hin*dIII and *Pst*I and hybridized after a Southern transfer with a labeled *bop* DNA fragment (Fig. 2B). Cleavage of the DNA isolated from the transformants as well as from untransformed cells with *Hin*dIII produced a smear in the high-molecular-weight range, which is due to the low cutting frequency of this enzyme in the halobacterial genome. The linearized vector, which has a size of 12.4 kb, was not visible after hybridization in the *Hin*dIII-cleaved DNA from the transformed L33-pEF191/1 and -/2 cells, indicating that the plasmid had integrated into the chromosome.

*Pst*I cuts the plasmid pEF191 into three fragments of 2.36, 2.67 and 7.37 kb, with the 2.67-kb fragment containing *bop*. In the DNA isolated from the transformants and cleaved with *Pst*I, two fragments were identified which hybridized with the *bop* sequence. The sizes of these fragments did not correspond to either the plasmid fragment or the L33 genomic *bop* fragment, indicating that homologous recombination into the chromosome had occurred at the *bop* locus. The *Pst*I digest allowed the determination of the side of the insertion element on which the integration had taken place: the L33-pEF191/2 transformant yielded DNA fragments of 3.45 kb and 4.86 kb, which resulted from homologous recombination upstream from the IS*H2* element. Insertion downstream from the IS*H2* element produced DNA fragments of 3.98 and 4.33 kb appearing as a doublet in the *Pst*I-digested DNA of L33-pEF191/1. Loss of the insertion element would reduce the size of one of the *Pst*I fragments in each transformant by 520 bp. This was not observed for either isolate. The comparison of *Pst*I-digested DNA from L33 with that from the *bop* wt strain R_1M_1 confirms that the increase in size caused by the IS*H2* integration can be well resolved, as is shown in Fig. 2B.

The mechanism of recombination depicted in Fig. 2A was confirmed by reprobing the Southern blot with labeled *E. coli* plasmid sequences (Fig. 2C). Besides the 4.33-kb fragment in L33-pEF191/1 and the 4.86-kb fragment in L33-pEF191/2 bearing *bop*, an additional band at 7.37 kb was detected in the transformants' DNA. This fragment corresponds to the plasmid fragment carrying Mev^R, the halobacterial *ori* as well as the complete *E. coli* plasmid sequence. This further supports the evidence that the identified transformants have incorporated the full length plasmid DNA by insertion at the chromosomal


A
L 33 :
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Pst I
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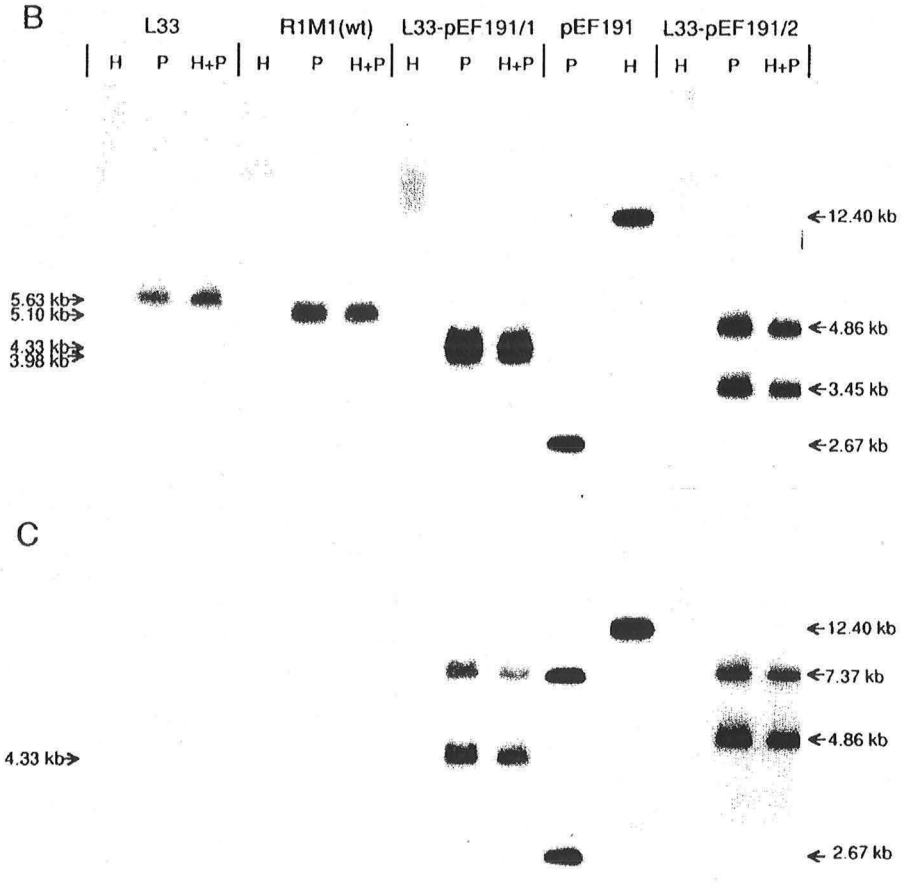
B
L33
R1M1(wt)
L33-pEF191/1
pEF191
L33-pEF191/2
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12.40 kb
5.63 kb
5.10 kb
4.33 kb
3.98 kb
4.86 kb
3.45 kb
2.67 kb
C
12.40 kb
7.37 kb
4.33 kb
4.86 kb
2.67 kb

bop locus. Integration occurred at one copy per chromosome, as determined by comparing the intensity of the hybridization signals from the DNA of the transformants with that of different molar amounts of digested plasmid (not shown). Internal crossover between the two copies of *bop* on the chromosome did not impair the stability of the genotype of the transformants after 12 months of continuous passage.

(c) Synthesis of BR in L33 cells transformed with pEF191

The ability to assay BR in the L33 transformants allowed us to investigate whether *bop* expression is dependent upon a defined upstream location of the regulatory genes *bat* and *brp*. Analysis of spontaneous *bop* mutants has demonstrated that *bat* and *brp* are involved in *bop* expression (Betlach et al., 1989), although nothing is known about the mechanism of interaction between these genes or their gene products. Since a *cis*-acting influence of the *bat* or *brp* sequence could not be excluded, Ni et al. (1990) developed a halobacterial *bop* expression vector which carried the entire *bop* cluster, with the exception of the last 43 bp distal to *bat*. This plasmid is the only construct currently available which produced wt levels of BR in *H. halobium*, but the state of the plasmid in transformed cells had not been investigated.

According to the recombination scheme depicted above, two different genomic configurations are obtained depending on the integration locus of the plasmid. If integration occurs upstream from the IS*H2* element, the wt *bop* cluster with the functional *bop* and the *bat* and *brp* in their native arrangement is restored. This has occurred in the transformant L33-pEF191/2. In the isolate L33-pEF191/1, which has the plasmid inserted downstream from the IS*H2* element, the expression of *bop* is driven by the promoter region provided on the plasmid pEF191. This region comprises only 388 bp upstream from the start codon. In this transformant, *bat* and *brp* are separated from the putative *bop* promoter by more than 10 kb, compared with 0.5 kb in the wt. In both transformants the second copy of the gene present on the chromosome remains inactivated by the insertion element IS*H2*.

We examined the level of *bop* expression of the two transformants L33-pEF191/1 and -/2 and compared it with the *bop* wt strain R_1M_1. Northern analysis demonstrates that the *bop* specific mRNA is synthesized at the same level in the wt (R_1M_1), the reconstituted (L33-pEF191/2) and the interrupted (L33-pEF191/1) *bop* cluster, whereas in the L33 recipient strain no *bop* mRNA is detectable (Fig. 3).

Purple membrane isolated from the transformants displayed the same absorption maximum and the same bouyant density on sucrose gradients as material isolated from the wt (Fig. 4 and data not shown). The amount of BR isolated from both transformants was 60–70 nmol per mg of total protein, and this value corresponded to the average amount that was produced by the wt.

These results demonstrate that the function of the *bop* promoter is independent of its location relative to the regulatory *bat* and *brp* sequences. These data are in agreement with the proposed regulation of *bop* via the gene products of *brp* and *bat*. The specific *brp* and *bat* mRNA transcripts have been determined under different conditions of growth, and a model for the function of these putative proteins has been proposed (Shand and Betlach, 1991).

The promoter region of *bop* has been defined by the determination of the transcription start point (Dunn et al., 1981) and the identification of adjacent conserved sequences (Thomm and Wich, 1988; Reiter et al. 1990). As BR synthesis in the transformant L33-pEF191/1 demonstrates, the 388-bp sequence upstream from the *bop* start codon contained in the plasmid pEF191 is sufficient

Fig. 2. Analysis of the homologous recombination. (**A**) Recombination between the homologous *bop* sequences on the plasmid (pEF191) and on the chromosome (L33) result in integration of the plasmid and duplication of the homologous DNA. Depending on the location of the integration point relative to the insertion element IS*H2*, contained in the chromosome of the recipient strain L33, two different configurations are obtained. Integration downstream from IS*H2* results in configuration 1, upstream from IS*H2* in configuration 2. The locations of the *Pst*I and *Hin*dIII restriction sites on the plasmid and the chromosome are indicated, as well as the size of the fragments that will arise after digestion of the depicted DNAs with these restriction endonucleases. The regions of homology on the chromosome and on the plasmid are indicated by variously hatched boxes. The pBR322 fragment used for Southern blot analysis is indicated by a black bar. The figure is not drawn to scale. (Panel **B**) Southern blot analysis of chromosomal DNA isolated from two independent transformants, L33-pEF191/1 and L33-pEF191/2, using the 1.4-kb *BamH*I-*Sal*I fragment of pEF1100 as a *bop*-specific probe. DNA from the untransformed recipient strain L33, from the *bop* wt strain R_1M_1, as well as an equimolar amount of pEF191 plasmid DNA were also analyzed. (Panel **C**) Rehybridization of the blot shown in panel **B** with pBR322 DNA. **Methods:** Isolation of total halobacterial DNA was performed as described by Vogelsang et al. (1983). After electrophoresis on an 1% agarose gel the DNA was transferred to a nitrocellulose membrane by capillary blotting (Sambrook et al., 1989) and hybridized with ^{32}P-labeled DNA fragments. Labeling of the DNA fragments was performed with the Random Primed DNA Labeling Kit from Boehringer Mannheim following the instructions of the manufacturer. Hybridization conditions were as follows: 0.1% SDS/5 × Denhardt's solution/2.5 × SSC/40% formamide/200 µg of sonified hering sperm DNA per ml hybridization solution and $1–2 \times 10^4$ cpm of the radioactive probe per cm^2 of membrane. After incubation at 40°C for 12–24 h, the filters were submitted to the following 15-min washes: 0.1% SDS/5 × Denhardt's solution/2.5 × SSC/40% formamide at 40°C; 2.5 × SSC/40% formamide at 40°C; 2.5 × SSC at room temperature. For the determination of the size of the labeled fragments on the Southern blots, the EtdBr-stained 1% agarose gel on which a molecular weight standard had been separated was photographed before being processed for blotting, and a print of the stained pattern was matched to the exact size of the nitrocellulose sheet and corresponding autoradiogram.

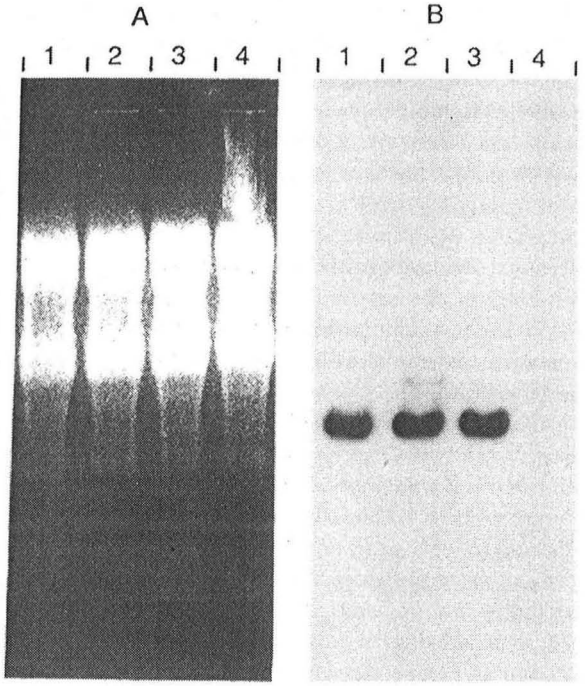

Fig. 3. Northern blot analysis of transcripts isolated from L33 cells transformed with pEF191. Total halobacterial RNA was isolated according to the procedure of Chomczynski and Sacchi (1987). Equal amounts of total RNA from L33-pEF191/2 (lane 1), L33-pEF191/1 (lane 2), R_1M_1 (lane 3), and L33 (lane 4), cells were electrophoresed through a 2.2 M formaldehyde-1% agarose gel (**A**), transferred to nitrocellulose and probed with a 1.4-kb DNA fragment bearing *bop* (**B**) as described in the legend to Fig. 2.

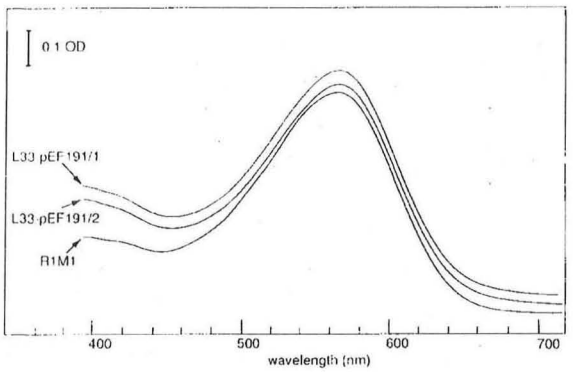

Fig. 4. Visible spectra of BR in membranes of transformed L33 cells and R_1M_1 wt cells. The arbitrary baseline was offset for clarity. **Methods:** The 700-ml cultures of L33 cells transformed with the *bop* expression vector pEF191 and of R_1M_1 wt cells were grown, harvested and dialyzed against distilled water overnight as described previously (Oesterhelt and Stoeckenius, 1974). Absorption spectra were recorded at room temperature in 10 mM Na-phosphate buffer at pH 7.0 using an Aminco DW2000 spectrophotometer. The amount of BR contained in the membrane preparation analysed was calculated from the absorption peak at 568 nm using $\varepsilon_{568\,nm} = 63000\ M^{-1}\ cm^{-1}$. The amount of total protein in the membrane preparations was determined with the BCA protein assay reagent following the protocol provided by Pierce.

for the expression of *bop*. This provides the first genetic approach to defining the limits of the *bop* promoter sequence in vivo. In the absence of a stable self-replicating *bop* expression vector for *H. halobium*, the site-specific integration described here may be further utilized to verify the putative transcription and translation signals of *bop*.

(d) Utilization of pEF191 for the construction and expression of BR mutants

The system we describe has also been used for the production and expression of site-directed *bop* mutants. Details about the mutant proteins will be given in a subsequent paper (U.S. and D.O., manuscript in preparation). The efficiency of mutagenesis using the procedure of Kunkel et al. (1987) varied between 40% and 60% depending on the number and on the location of the mutated positions. The mutations we have introduced affected the absorption behaviour of the pigment, and the halobacterial colonies expressing the mutated *bop* could be distinguished from those in which vector integration had restored the wt gene by colour selection. The frequency with which transformants expressing the wt gene were obtained was not systematically determined but was dependent on the location of the mutation relative to the IS*H2* in the genomic copy of the gene. The genomic configuration as well as the presence of the desired mutation on the chromosomal DNA of the transformants can also be easily established by PCR techniques.

The absorption spectrum of a mutated BR obtained with the pEF191 vector is shown in Fig. 5. In this mutant (D85T) the Asp^{85} has been changed to a Thr, which results in a 19-nm red shift of the absorption maximum. Since this mutation is located only 50 bp downstream from the position of IS*H2* on the chromosome, the geno-

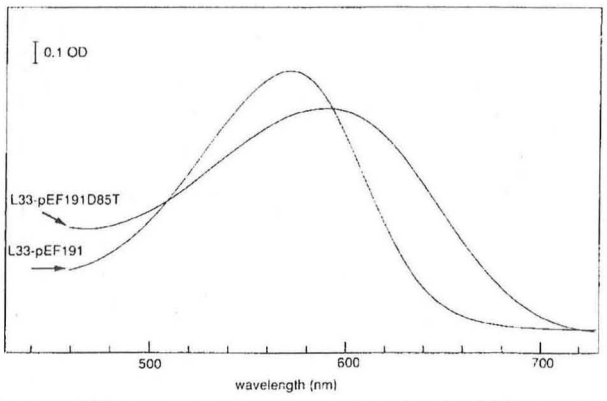

Fig. 5. Visible spectra of L33 cells transformed with pEF191 bearing the wt and a mutated *bop*. In the mutant BR (L33-pEF191D85T), Asp^{85} has been changed to Thr. This resulted in a red shift of the absorption maximum of 19 nm. The spectra were recorded under the same conditions as described in the legend to Fig. 4.

mic arrangement in which the wt gene is restored and the mutated gene is inactivated by the IS*H2* requires that the strand break leading to vector integration occur within this 50-bp sequence between the mutation and IS*H2*. The probability of this event is low compared with recombination over the whole 780-bp region of homology. In the transformation experiment performed with this plasmid, restoration of the wt phenotype was not observed, confirming this assumption.

In order to exploit halobacteria as a novel expression system, further tailoring of the vector pEF191 will be performed, which will facilitate the cloning and expression of other genes coding for seven transmembrane helical proteins and for other retinal proteins in particular.

Note: As this paper was submitted for review, a paper by Mankin et al. (1992) was published, which reports on the mutagenesis of the 23S RNA gene of *H. halobium* via homologous recombination of plasmid sequences into the genome.

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