Bacterio-opsin mutants of *Halobacterium halobium*

(purple membrane/bacteriorhodopsin/cloning/insertion/operon)

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ABSTRACT The bacterio-opsin (*bop*) gene of *Halobacterium halobium* R1 has been cloned with about 40 kilobases of flanking genomic sequence. The 40-kilobase segment is derived from the (G+C)-rich fraction of the chromosome and is not homologous to the major (pHH1) or minor endogenous covalently closed circular DNA species of *H. halobium*. A 5.1-kilobase *Pst* I fragment containing the *bop* gene was subcloned in pBR322 and a partial restriction map was determined. Defined restriction fragments of this clone were used as probes to analyze the defects associated with the *bop* gene in 12 bacterio-opsin mutants. Eleven out of 12 of the mutants examined had inserts ranging from 350 to 3,000 base pairs either in the *bop* gene or up to 1,400 base pairs upstream. The positions of the inserts were localized to four regions in the 5.1-kilobase genomic fragment: within the gene (one mutant), in a region that overlaps the 5' end of the gene (seven mutants), and in two different upstream regions (three mutants). Two revertants of the mutant with the most distal insert had an additional insert in the same region. The polar effects of these inserts are discussed in terms of inactivation of a regulatory gene or disruption of part of a coordinately expressed operon. Given the defined nature of the *bop* mRNA—i.e., it has a 5' leader sequence of three ribonucleotides—these observations indicate that the *bop* mRNA might be processed from a large mRNA transcript.

The purple membrane of *Halobacterium halobium* is one of the best-characterized natural membranes (1). This membrane functions as a light-driven proton pump and contains one major protein, bacterio-opsin, bound stoichiometrically to the chromophore, retinal (2). The complex of bacterio-opsin and retinal is referred to as bacteriorhodopsin. The amino acid sequence of bacterio-opsin has been determined (3, 4). A *Pst* I-derived fragment of *H. halobium* strain S9 has been cloned, and the sequences of the structural gene for bacterio-opsin (*bop*) and its flanking regions have been determined (5). The nucleotide sequence is in agreement with the amino acid sequence with the exception of an additional 13-residue amino-terminal component, and one additional carboxyl-terminal residue. The major *bop* mRNA component isolated from this strain extends only three nucleotides 5' to the start codon (5, 6).

The total cellular content of bacterio-opsin is variable over a 4- to 5-fold range with respect to growth conditions (7). At low oxygen levels with illumination, the purple membrane can represent as much as 50% of the cell surface area (1, 8). Mutants of the wild-type strain that "overproduce" purple membrane and bacterio-opsin have been isolated (e.g., *H. halobium* S9) (9). However, there is no direct evidence for transcriptional regulation of bacterio-opsin synthesis. Mutants defective for purple membrane (Pum), ruberin synthesis (Rub), and gas vacuole formation (Vac) occur at frequencies of 10^{-2} – 10^{-4} (10). About 50% of the Pum mutants lack bacterio-opsin while the remainder are defective in the synthesis of retinal. The Bop[−] mutants are rather

stable, but revertants have been recovered from one mutant.

We report here the cloning of a *Pst* I fragment (P5.1) containing the *bop* gene of *H. halobium* R1, a Vac[−] derivative of wild type. We have determined a partial restriction site map of the P5.1 clone and most of the nucleotide sequence of the *bop* gene. No differences have been noted yet between the coding sequence of the *bop* gene of this clone and the one previously reported. We used the P5.1 clone and defined restriction fragments of the clone as probes to characterize a number of Bop[−] mutants and two revertants of one of these mutants. In all cases except one, the Bop[−] mutants have additional DNA segments, ranging from 350 to 3,000 base pairs (bp), in the P5.1 fragment. One mutant clearly has an insert in the *bop* structural gene. The majority of the mutants have inserts in a 290-bp region that overlaps the amino-terminal coding segment of the *bop* gene. Whether or not any of these inserts are located either in the gene or immediately upstream has not yet been determined. One mutant has an insert in a region 470–620 bp upstream of the *bop* gene. Two mutants have inserts in a region 1,200–1,500 bp upstream of the gene. Two revertants of one of the mutants in this latter location have an additional small insert near or in the original insert. The implications of these observations are discussed.

MATERIALS AND METHODS

Materials. [γ -³²P]ATP (carrier free) and [α -³²P]dNTPs were from Amersham. Restriction endonucleases, T4 polynucleotide kinase, and DNA polymerase I were from New England Biolabs. Bacterial alkaline phosphatase and DNase I were obtained from Worthington. Reverse transcriptase (RNA-dependent DNA polymerase) from avian myeloblastosis virus was obtained from J. Beard (Life Sciences, St. Petersburg, FL). Dideoxynucleoside triphosphates were from P-L Biochemicals. The 15-nucleotide-long primer specific for a DNA sequence in the *bop* gene was synthesized by T. Horn of Genentech (South San Francisco, CA). FI and FII fractions of *H. halobium* DNA were provided by K. Ebert (Wurzburg, Federal Republic of Germany).

Strains and Growth Conditions. All halobacterial strains used are listed in Table 1. Halobacterial strains were grown in salt medium [4 M NaCl/0.12 M MgSO₄/30 mM KCl/10 mM trisodium citrate/0.5% Casamino acids/1% peptone (Inolex), pH 7.2] with shaking and illumination at 37°C for 4 to 5 days.

Purification of DNA. *Escherichia coli* plasmid DNA was isolated by the alkaline NaDodSO₄ method (13). Halobacterial total genomic and covalently closed circular (ccc) DNAs were isolated in the following manner. Cultures of halobacteria (500 ml) were harvested in late logarithmic phase by centrifugation, suspended in 6 ml of basal salt (4 M NaCl/0.12 M MgSO₄/30 mM KCl/10 mM trisodium citrate, pH 7.2) each, and lysed by ad-

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Abbreviations: kb, kilobase(s); bp, base pair(s); ccc DNA, covalently closed circular DNA.

Table 1. *H. halobium* strains used

Strain	Phenotype	Source	Ref.
Wild type	Vac ⁺ Rub ⁺ Pum ⁺	D. Oesterhelt	9, 10
Mutant			
Mutagenized			
S9	Vac ⁻ Rub ⁺ Pum ⁺⁺	W. Stoeckenius	9
S90D2*	Vac ⁻ Rub ⁺ Pum ⁻	J. Spudich	
S9Flx3*	Vac ⁻ Rub ⁺ Pum ⁻	J. Spudich	
JN2	Vac ⁻ Rub ⁺ Pum ⁻	H. J. Weber	
ET-15	Vac ⁻ Rub ⁺ Pum ⁻	H. J. Weber	
ET-1001	Vac ⁻ Rub ⁺ Pum ⁺⁺	W. Stoeckenius	
Spontaneous			
R1	Vac ⁻ Rub ⁺ Pum ⁺	W. Stoeckenius	11
IV-3	Vac ⁻ Rub ⁺ Pum ⁻	G. Weidinger	10, 12
IV-4	Vac ⁻ Rub ⁺ Pum ⁻	G. Weidinger	10, 12
reIV-41	Vac ⁺ Rub ⁺ Pum ⁺	F. Pfeifer	
reIV-42	Vac ⁺ Rub ⁺ Pum ⁺	F. Pfeifer	
IV-5	Vac ⁻ Rub ⁺ Pum ⁻	G. Weidinger	10
IV-8	Vac ⁻ Rub ⁺ Pum ⁻	G. Weidinger	10
IV-9	Vac ⁻ Rub ⁺ Pum ⁻	G. Weidinger	10
IV-10	Vac ⁻ Rub ⁺ Pum ⁻	G. Weidinger	10
IV-11	Vac ⁻ Rub ⁺ Pum ⁻	G. Weidinger	10
IV-12	Vac ⁻ Rub ⁺ Pum ⁻	G. Weidinger	10
IV-13	Vac ⁻ Rub ⁺ Pum ⁻	G. Weidinger	10
Vac ⁻ and Rub ⁻	Vac ⁻ Rub ⁻ Pum ⁺	F. Pfeifer	10, 12

*S90D2 is a Bop⁻ halorhodopsin overproducer recovered from a mutagenized culture of S9. S9Flx3 is a Bop⁻ halorhodopsin⁻ mutagenized derivative of S90D2.

dition of 20 μ l of 4% sodium desoxycholate (30 min, 0°C). After centrifugation (10,000 $\times$ g, 15 min, 0°C), the cleared lysate was diluted with 2 vol of water and the DNA was precipitated by addition of polyethylene glycol 6000 to a final concentration of 10% (4°C, 5 hr). The pellet was dissolved in 50 mM NaCl/5 mM EDTA/10 mM Tris-HCl, pH 8.0, and subjected to cesium chloride/ethidium bromide gradient centrifugation (Ti 50 rotor, 42,000 rpm, 2°C, 20 hr). The ccc DNA and genomic DNA bands were collected separately, ethidium bromide was removed with isopropanol, and the DNA was dialyzed against 10 mM Tris-HCl, pH 8.0/1 mM EDTA.

Cloning of the Bacterio-opsin Gene. A cosmid bank of *H. halobium* strain R1 total DNA was made by the Ish-Horowitz procedure (14, 15). A partial *Sau*3A I restriction digest was fractionated on a 5–40% sucrose gradient (SW 27 rotor, 26,000 rpm, 13 hr, 20°C) and fractions containing 35- to 40-kilobase (kb) fragments (0.4 μ g) were treated with bacterial alkaline phosphatase and ligated to 1 μ g of *Bam*HI-digested cosmid vector pJB8 (14). The ligated molecules were packaged *in vitro* and transfected into *E. coli* HB101 (*recA*⁻, *pro*⁻, *leu*⁻, *B*₁⁻, *r*_B⁻*m*_B⁻), and ampicillin-resistant colonies were selected on agar medium. One thousand ampicillin-resistant clones were screened by colony filter hybridization (14) with an [α -³²P]-labeled 22-nucleotide cDNA as probe. The short cDNA probe was made by extension of a 15-base-long synthetic oligonucleotide primer [residues 456–470 of the reported *bop* gene sequence (5)] using reverse transcriptase and partially purified mRNA (6, 16) from *H. halobium* strain ET1001 (*bop* overproducer) as template (17). The oligonucleotide was extended with [α -³²P]dNTPs, to the first adenine residue in the mRNA sequence to yield a specific cDNA of 22 nucleotides that was subsequently purified on a 20% acrylamide/7 M urea gel (18).

Nick-Translation and Southern Transfer. Gel-purified DNA restriction fragments were labeled according to Rigby *et al.* (19). Prior to hybridization, DNA probes were denatured in 0.4 M sodium hydroxide for 10 min at 80°C. DNA (2 μ g) was digested with restriction endonuclease and fractionated on 1%, 1.5%, or

2% agarose gels (10) and transferred to S & S nitrocellulose filters by using the procedure of Southern *et al.* (20). Filters were incubated for 2 hr at 41°C in 0.75 M NaCl/0.075 M Na citrate, pH 7/0.1% polyvinylpyrrolidone/0.1% Ficoll/1.0% bovine serum albumin (20)/0.2 M sodium phosphate buffer, pH 6.5/50% formamide containing 100 μ g of *E. coli* tRNA (Sigma) per ml. Hybridizations were performed in sealed plastic bags using 0.01 μ g of [α -³²P]DNA per ml (10⁶–10⁷ cpm) in the above hybridization mixture. After hybridization at 41°C for 20 hr, the filters were washed four times with 0.30 M NaCl/0.03 M Na citrate, pH 7/0.5% NaDodSO₄ at room temperature and twice with 15 mM NaCl/1.5 mM Na citrate, pH 7/0.5% NaDodSO₄ at 58°C over a 2-hr period. Kodak X-Omat AR5 films were used for autoradiography (–70°C, 3–24 hr with Dupont Cronex Lightning plus screens).

RESULTS

Characterization of the *bop* Gene Clone. Two cosmid clones ($\approx$ 40 kb each) from a sample of 1,000 genomic clones hybridized with the bacterio-opsin cDNA probe. *Pst* I digestion of these cosmids yielded 9 or 10 fragments from each with a common set of 6 fragments, suggesting that these two cosmids contain overlapping segments of the genome. Southern blot analysis with the cDNA probe identified a 5.1-kb *Pst* I fragment from each cosmid. The 5.1-kb fragment (P5.1) was subcloned in the *Pst* I site of pBR322 (21). Diagnostic restriction enzyme digestion and nucleotide sequence analysis (data not shown) confirmed the presence of the *bop* gene on the P5.1 fragment. A partial restriction site map of the P5.1 fragment is presented in Fig. 1.

Localization of the *bop* Gene in the Genome of *H. halobium*. The total DNA of *H. halobium* can be separated by column chromatography into two components on the basis of G+C content. A major fraction (fraction 1) is 68% G+C and a "satellite fraction" (fraction 2) is 58% G+C (22, 23). ccc DNAs are found in fraction 2 along with chromosomal DNA (12). *Pst* I digests of total DNA of wild-type *H. halobium* and fractions 1 and 2, as well as purified ccc DNAs [main species pHH1, a DNA of 100 megadaltons, and several minor ccc DNA species (24)], were subjected to Southern blot analysis and hybridized to the P5.1 fragment and one of the cosmid clones (Fig. 2). Fraction 1 of *H. halobium* DNA, when digested with *Pst* I and probed with total *bop* cosmid DNA, has 11 hybridizing fragments, most of which are equivalent in size to the *Pst* I fragments of the cosmid, including the P5.1 fragment. No significant hybridization was observed in *Pst* I digests of fraction 2 DNA. The same results were obtained when the P5.1 fragment was used as a probe (Fig. 2A). *Pst* I digests of total *H. halobium* DNA and purified ccc DNA probed with the P5.1 fragment yielded one 5.1-kb fragment in the former and no significant signal with the latter (Fig. 2B). The cosmid containing the *bop* gene must have been derived from a contiguous 40-kb region of the genome of overall high G+C content that has no homology to fraction 2 DNA or the ccc DNA of *H. halobium*. The observation that the P5.1 *bop* fragment has no homology with the ccc DNA is relevant to the observation that Bop⁻ mutants usually have coincident gross alterations of the pHH1 ccc DNA (10).

Comparison of *Pst* I digests of total DNA from wild-type *H. halobium* and mutants R1 and S9 with the P5.1 probe revealed a single 5.1-kb hybridizing fragment in each case (Fig. 2C). *H. halobium* R1 is a vacuole-deficient (Vac⁻) derivative of wild type, and S9 is a bacterio-opsin overproducer derivative obtained from a mutagenized culture of R1 (9). Fourteen independently isolated Vac⁻ and/or Rub⁻ (ruberin-deficient) Pum⁺ mutants (10) have a 5.1-kb *Pst* I fragment hybridizing with the P5.1 cloned fragment.

FIG. 1. Partial restriction site map of the P5.1 fragment. Not all *Nae* I and *Bst*NI sites are shown. The heavy black bar indicates the *bop* gene coding sequence. Arrows mark regions where insertions occur: 1, a 300-bp region defined by *Bam*HI and *Nae* I sites; 2, a 150-bp region defined by *Nae* I and *Ava* I sites; 3, a 290-bp region defined by *Nae* I and *Ava* I sites; 4, a 429-bp region defined by *Ava* I sites. Internal DNA restriction fragments that were isolated and used as probes in the Southern analysis are represented beneath the restriction map.

Southern Blot Analyses of *Bop*⁻ Genomes. *Bop*⁻ mutants are derived spontaneously from either *Vac*⁻ or *Rub*⁻ *H. halobium* at a frequency of 10^{-4} . Some *Bop*⁻ mutants were obtained from mutagenized cultures of *H. halobium* (Table 1). DNAs from 12

FIG. 2. Localization of the *bop* gene fragment in the genome of *H. halobium*. (A) *Pst* I restriction enzyme digests of fraction I (lane 5) and fraction II (lane 6) DNAs were electrophoresed on a 1% agarose gel as described (9). The digests (fraction 1, lanes 1 and 3; fraction 2, lanes 2 and 4) were transferred to nitrocellulose and probed with the nick-translated P5.1 fragment (lanes 3 and 4) and one of the two cosmid clones (lanes 1 and 2). (B) Southern blots of *Pst* I-digested wild-type total DNA (lane 3) and isolated ccc DNA (lane 4) were hybridized to the P5.1 probe (total DNA, lane 1; ccc DNA, lane 2). (C) Southern blots of *Pst* I-digested total DNA from various strains of *H. halobium* were probed with the P5.1 fragment. Lanes: 1–3, wild-type, S9, and R1, respectively.

Bop⁻ mutants were digested with *Pst* I and probed in Southern blots with the P5.1 fragment. Eleven of the 12 mutants have a larger fragment in lieu of the 5.1-kb fragment when hybridized with the P5.1 probe. One mutant has a *Pst* I fragment equivalent in size to the wild-type *Pst* I fragment. The sizes of the fragments in these 11 mutants are 350–3,000 bp larger than the 5.1-kb wild-type fragment. These observations suggest that the 11 *Bop*⁻ mutants have incurred insertions of DNA in or near the *bop* structural gene.

The locations of the insertions in the P5.1 fragment of these mutants were defined by a series of Southern blot analyses of restriction endonuclease-digested total genomic DNAs probed with defined segments of the P5.1 clone (Fig. 1). Eight of the insertions were localized to the *Bam*HI/*Bst*EII 1,640-bp segment. In each case, the genomic *Bam*HI/*Bst*EII hybridizing fragment was larger by an increment of the same size as found for the respective *Pst* I fragments. The other three mutants had *Bam*HI/*Bst*EII hybridizing fragments equivalent in size to the wild-type fragment, clearly indicating that their inserts are not located in the *bop* gene. These three mutants had inserts in the *Bam*HI 1,050-bp fragment upstream to the *bop* gene (Fig. 3A, lanes 2, 8, and 11).

The locations of the insertions in all of the 11 mutants were narrowed to four regions of the P5.1 fragment (Fig. 1 and Table 2). Two of the regions (nos. 2 and 4) have only one representative insertion event. The location of the 350-bp insert in the JN2 mutant was defined by a Southern blot analysis of an *Ava* I digest of JN2 genomic DNA probed with the *Bam*HI/*Bst*EII fragment. An *Ava* I 780-bp fragment hybridized with the probe

FIG. 3. Southern blot analyses localizing inserts of *Bop*⁻ mutants in regions 1 and 2. (A) Southern blots of *Bam*HI-digested genomic DNAs were probed with the 1,050-bp *Bam*HI fragment. Wild-type (lanes 6 and 12) has one fragment of 1,050 bp hybridizing to the probe. Lanes: 1, S90D2; 2, IV-12; 3, IV-11; 4, IV-10; 5, IV-9; 7, reIV-41; 8, IV-4; 9, IV-13; 10, IV-8; 11, IV-3; 13, reIV-42. (B) Southern blots of *Nae* I-digested genomic DNAs were probed with the 450-bp *Nae* I fragment. Wild-type (lane 1) has one fragment of 450 bp hybridizing to the probe. Lanes: 2, IV-3; 3, IV-4; 4, reIV-41; 5, IV-10. The larger fragments on this gel were not accurately measured because they were located in a nonlinear area of the gel. (C) Southern blots of *Bst*NI-digested genomic DNAs were probed with the 390-bp *Nae* I fragment. Wild-type (lane 1) has one hybridizing fragment of 1,090 bp. Lanes: 2, IV-4; 3, IV-12; 4, IV-8.

Table 2. Summary of Bop⁻ mutant and revertant analysis

	Region of insertion*	Insertion size, bp
Mutant		
IV-3	1	600
IV-4	1	3,000
IV-12	2	450
IV-8	3	1,100
IV-9	3	1,100
IV-10	3	900
IV-11	3	1,100
IV-13	3	1,100
S90D2	3	1,100
S9Flx3	3	1,100
ET-15	3	1,100
JN2	4	350
Revertant		
reIV-41	1	350
reIV-42	1	350

* See Fig. 1.

in lieu of the wild-type *Ava* I 429-bp fragment (Fig. 4A, lane 7), which defines the location of the insert to the *Ava* I 429-bp segment (region 4, Fig. 1) of the *bop* structural gene. This is the only mutant of the series analyzed in this report that has an unequivocal location of the insert in the structural gene. The insert of mutant IV-12 is located in region 2. This mutant has a wild-type *Ava* I 530-bp fragment (Fig. 4A, lane 13) and an enlarged

FIG. 4. Southern blot analyses localizing inserts of Bop⁻ mutants in regions 3 and 4. (A) Southern blots of *Ava* I-digested total DNAs were hybridized to the nick-translated 1,640-bp *Bam*HI/*Bst*EII fragment (Fig. 1). Lanes 1 and 14, wild-type *H. halobium*, wild-type *Ava* I fragments hybridizing to this probe are 429, 530, and 1,150 bp; 2, ET-15; 3, S90D2; 4, S9Flx3; 5, IV-9; 6, IV-10; 7, JN2; 8, reIV-41; 9, IV-4; 10, reIV-42; 11, IV-8; 12, IV-3; 13, IV-12. (B) Southern blots of *Nae* I-digested total DNAs were hybridized to the nick-translated 390-bp *Nae* I fragment. Wild-type (lane 1) has fragments of 390 bp, 950 bp (partial), and 1,340 bp (partial) hybridizing to the probe. Lanes: 2, S90D2; 3, IV-8; 4, IV-10. (C) Southern blots of *Nae* I-digested total DNAs were probed with the nick-translated 429-bp *Ava* I fragment. Wild-type (lanes 1 and 5) has one fragment of 950 bp hybridizing to the probe. Lanes: 2, S90D2; 3, IV-8; 4, IV-10; 6, ET-15; 7, IV-13; 8, IV-11.

*Bam*HI 1,050-bp fragment (Fig. 3A, lane 2). The wild-type *Bst*NI 1,090-bp fragment (Fig. 3C, lane 1) is replaced with a fragment of 1,540 bp (lane 3), indicating that the 450-bp insert is between the *Bst*NI and *Ava* I sites overlapping region 2. Additional analysis using *Nae* I-digested IV-12 DNA probed with the *Nae* I 390-bp fragment localized the insert to that 150-bp region defined by the *Nae* I and *Ava* I sites of region 2 upstream of the *bop* gene (data not shown).

Mutants IV-8, IV-9, IV-10, IV-11, IV-13, S90D2 (and its derivative S9Flx3), and ET-15 have inserts in region 3, which overlaps the first 30 codons of the 5' terminus of the *bop* gene. All of these mutants have altered *Ava* I 530-bp fragments (Fig. 4A). ET-15 is representative of several similar mutants (IV-11, IV-13, ET-15) that have inserts of 1,100 bp containing an internal *Ava* I site. In these mutants, the *Ava* I 530-bp fragment is absent and a 480-bp *Ava* I fragment is present (Fig. 4A, lane 2). It is likely that the 1,100-bp insertion is near one of the *Ava* I sites of the 530-bp fragment and that there is an internal *Ava* I site asymmetrically located near the edge of the insert. This insert would generate a smaller *Ava* I 530-bp fragment and another fragment with too little homology to be detected by the *Bam*HI/*Bst*EII 1,640-bp probe. The other four mutants (IV-8, IV-9, IV-10, S90D2) that have altered *Ava* I 530-bp fragments show a loss of the 530-bp fragment as well as the appearance of either a 1,630-bp fragment (Fig. 4A, lanes 3, 4, 5, and 11) or a 1,400-bp fragment (lane 6). These size increases correspond to insert sizes of 1,100 and 900 bp. *Nae* I digests of total DNA of all of the mutants having altered *Ava* I 530-bp fragments were hybridized with either the *Nae* I 390-bp probe (Fig. 4B) or the *Ava* I 429-bp probe (Fig. 4C). Signals corresponding to wild-type were seen with the *Nae* I 390-bp probe, indicating the absence of inserts to the left of the internal *Nae* I site of the wild-type *Ava* I 530-bp fragment. Faint larger partial digestion products (>1,340 bp) on this blot not corresponding in size to wild-type partials contain the *Nae* I fragment bearing the insert. Hybridization with the *Ava* I 429-bp probe (Fig. 4C) places the inserts between the *Ava* I site near to the 5' end of the *bop* gene and the first upstream *Nae* I site (region 3). Inserts of two sizes occur in this region and several carry an *Ava* I site, which suggests that at least three unique insert sequences can violate this region.

The inserts of two mutants, IV-3 and IV-4, were mapped to region 1 (Fig. 3B, lanes 2 and 3). The IV-4 insert has an *Nae* I site generating two *Nae* I fragments hybridizing to the *Nae* 450-bp probe (lane 3). The third fragment should represent the partial with the internal *Nae* I site uncut. The revertant, reIV-41, has an altered *Nae* I fragment pattern (lane 4) relative to its parent IV-4, which can be interpreted as an additional insertion of about 400 bp either within the original insert or very close to it. The two revertants (reIV-41 and reIV-42) appear to be similar (data not shown).

DISCUSSION

Eleven out of 12 Bop⁻ mutants examined in this study have insertions either in the structural gene or in specific regions of DNA upstream of the structural gene. These observations are not too surprising considering previous reports that *H. halobium* has a large number of repetitive sequence families (at least 50) with apparently high frequencies of transpositions (25, 26) and that the ccc DNA of *H. halobium* contains multiple insertions occurring concurrently with spontaneous phenotypic mutations (10). Preliminary analysis of three of the insertions (IV-4, reIV-41, IV-8) indicates that they lack homology and belong to different insertion families.

It seems unlikely that the Bop⁻ phenotype in these 11 mutants is due to a concurrent point mutation in the *bop* gene rather

than the insertion events identified in the P5.1 fragment. First, $\text{Vac}^- \text{Pum}^+$ and $\text{Rub}^- \text{Pum}^+$ mutants (10) have no inserts in the P5.1 fragment and, second, the two revertants of the mutant with the most distal insert have an additional insert in the same region. Since the spontaneous mutation frequency to Pum^- in *H. halobium* is so high (10^{-4}) and is associated largely with insertion events, the Bop^- mutants obtained from mutagenized cultures (listed as Pum^- in Table 1) probably result from an insertion event rather than a nucleotide change. The nature of the mutational event in the Bop^- mutant (IV-5), which has no detectable insert in the P5.1 fragment, has not yet been determined.

The fact that the majority of *bop* mRNA transcripts have a three-nucleotide 5' leader sequence must be reconciled with the loss of bacterio-opsin synthesis as a result of upstream insertion events (500–1,400 bp upstream). One possible model to explain these observations is that the upstream inserts (regions 1 and 2) might inactivate a regulatory gene whose product activates a promoter contiguous to the *bop* gene. The negation of the IV-4 insert via a second insertion event could be explained by assuming that the original insert inactivated a promoter sequence for the putative regulatory gene and that the secondary insertion event provided a new promoter.

Another possible model is that the *bop* gene message is part of a larger transcript that initiates at an upstream promoter and proceeds through the *bop* gene with specific mRNA processing leading to the mature *bop* mRNA. Preliminary experiments have detected RNA transcripts of the *bop* gene larger than the mature mRNA species. Insertion elements could disrupt the upstream transcript or alter its overall secondary structure in such a way as to obviate processing (or possibly facilitate improper processing). The additional insert in the revertant could be explained as suggested above for the regulatory gene model, or it could eliminate a termination signal or restore the proper secondary structure required for processing. This model raises questions concerning the nature of the unusually large (at least 1,500-nucleotide) putative upstream transcript. It seems unlikely that this transcript serves only as a long leader sequence that is subsequently removed. If this model is proven correct, we suggest that the DNA upstream of the *bop* gene defines an additional gene that is coordinately expressed with the *bop* gene. Thus, the *bop* gene could be part of an operon containing at least one other, as yet unidentified, structural gene.

Note Added in Proof. H. Vogelsang and D. Oesterhelt have also observed inserts associated with Bop^- mutants (personal communication).

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