Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 2201–2205, April 1983
Biochemistry

High-frequency spontaneous mutation in the bacterio-opsin gene in *Halobacterium halobium* is mediated by transposable elements

(purple membrane/retinal/proton translocation/site-specific insertion)

SHILADITYA DASSARMA*, UTTAM L. RAJBHANDARY*, AND H. GOBIND KHORANA*†

Departments of *Biology and †Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Contributed by H. Gobind Khorana, January 20, 1983

ABSTRACT **We have recently characterized a transposable element, ISH*1*, which inactivates the bacterio-opsin (BO) gene in two purple membrane-deficient (Pum$^-$) mutants of *Halobacterium halobium*. Examination of nine additional Pum$^-$ mutants now shows that in all of these the BO gene has been inactivated by insertion of one of two types of transposable elements. Four Pum$^-$ strains contain ISH*1* within the BO gene, probably at the same site that we have previously characterized. A second element, ISH2, which is present in four more strains, inserts at multiple sites within the BO coding sequence. Significantly, another Pum$^-$ strain contains the ISH2 element 102 nucleotides upstream from the initiator codon for BO. ISH2, which is 520 nucleotides long, is the smallest insertion sequence known. Its sequence has been determined: it is A+T-rich (53%), contains a 19-base-pair inverted repeat at its termini, and, interestingly, duplicates either 10 or 20 base pairs at the target site during insertion. ISH2 is present in multiple copy numbers in the genome and contains several relatively short open reading frames.**

The purple membrane carries out light-dependent vectorial translocation of protons in *Halobacterium halobium* (1). It contains a single protein, bacterio-opsin (BO), whose amino acid sequence has been determined by analysis of both the protein and the gene (2, 3). *All-trans*-retinal serves as the chromophore and is linked as a Schiff base to the ε-amino group of lysine-216 (4) in the protein. Purple membrane-deficient (Pum$^-$) mutants arise spontaneously at a high frequency ($\approx 10^{-4}$) (5), and we have recently characterized two Pum$^-$ mutants in which the BO gene has been inactivated by the insertion of a transposable element, ISH*1*. In these mutants, ISH*1* was shown to insert at the same site but in opposite orientations. In addition, several structural features of ISH*1*, including the nucleotide sequence, were documented (6).

In a further investigation of the role of insertion elements in inactivation of the BO gene, we have now studied nine additional Pum$^-$ mutants of *H. halobium*. We find that, in all of these mutants, the BO gene is inactivated by insertion of one of two transposable elements. Four of these strains contain ISH*1* within the BO gene, the element being probably present at the same site as identified previously (6). Four more strains contain a second element, ISH2, which is present at multiple sites within the BO gene. In another strain ISH2 is present 102 nucleotides upstream from the initiator methionine codon for BO. In this paper we document the above findings and provide characterization of ISH2.

MATERIALS AND METHODS

Materials. The sources of enzymes and radiolabeled compounds were as previously described (6).

Strains. *H. halobium* strain S9, Pum$^+$, forms purple colonies on agar plates, and Pum$^-$ derivatives were identified by the orange color of the colonies. Seven independent spontaneous Pum$^-$ strains were isolated by screening approximately 35,000 colonies. These mutant strains are SD3, SD9, SD10, SD12, SD16, SD17, and SD19; SD17 has been described previously (6).

Three other Pum$^-$ strains were gifts. Strain L33 was obtained from J. Lanyi (7), strain OD2 was from J. Spudich (8), and strain R1mR was from Y. Mukohata (9).

Cloning. Total DNAs from *H. halobium* Pum$^+$ strain R1 and Pum$^-$ strains SD19, L33, and R1mR were cleaved with *Bam*HI and fractionated on a 1% low-melting agarose gel. Fragments 6–8 kilobase pairs (kbp) long [identified by Southern hybridization (10) as containing the BO gene] were eluted and inserted into the *Bam*HI site of pBR322 (6). Colonies were screened by using the BO gene-specific probe (see below). Approximately 1% of the colonies containing recombinant plasmids showed a positive hybridization response.

Cloned BO genes were identified as a 6.4-kbp *Bam*HI fragment for strain R1 and as a 6.9-kbp *Bam*HI fragment for strains SD19, L33, and R1mR. Restriction analysis of the cloned 6.9-kbp fragment from each of the latter strains confirmed the presence of a common insert, approximately 500 bp, designated as ISH2. This insert in all cases contained two *Bgl* II sites (the remainder of *H. halobium* DNA and the pBR322 vector has no *Bgl* II site). These two ISH2-specific *Bgl* II sites, which are 170 bp apart, were used to prepare an ISH2-specific probe (see Fig. 1 and below).

Probes for Southern Hybridization. *Probe 1, BO gene-specific.* This is a cloned 80-bp cDNA fragment that corresponds to the NH_2-terminal coding region of the BO gene (3, 11). *Probe 2, Bam*HI/Bst*EII fragment.* This is a 1.6-kbp *Bam*HI/*Bst*EII fragment that contains the entire coding region of the BO gene and approximately 800 bp of flanking sequences, 400 bp upstream and about the same length downstream (Fig. 1). *Probe 3, ISH1-specific.* This is a 1-kbp *Tth* I fragment from within the element in pSD17 (6). *Probe 4, ISH2-specific.* This is a 170-bp *Bgl* II fragment from within the ISH2 element in R1mR (Fig. 1) (see above). Probes 1, 3, and 4 were labeled by the method of O'Farrell (12) and probe 2 was labeled by nick-translation (13).

RESULTS

Ten Independently Isolated Pum$^-$ Strains Contain Insertions Within the BO Coding Region. *Xma* III digests of DNA from Pum$^+$ and Pum$^-$ mutants were analyzed by Southern blot hybridization using the BO gene-specific probe. As seen in Fig.

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Abbreviations: ISH, insertion elements from *Halobacterium halobium*; BO, bacterio-opsin; Pum, purple membrane phenotype; bp, base pair(s); ORF, open reading frame.

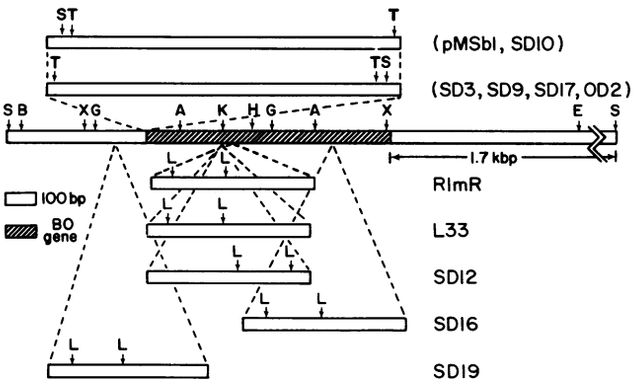

FIG. 1. Sites of insertion into and partial restriction map of the BO gene region for strains of *H. halobium*. The map at the center is for the *Sma* I fragment from Pum$^+$ S9. The BO gene is shown hatched. Insertions of ISH*1* at a single site in the NH_2-terminal coding region are indicated above in either one orientation for pMSb1 and SD10 or the opposite orientation for SD3, SD9, SD17, and OD2. Insertions of ISH2 at multiple sites in the BO structural gene for R1mR, L33, SD12, and SD16 or further upstream for SD19 are shown below. Restriction endonuclease sites: A, *Ava* I; B, *Bam*HI; G, *Bgl* I, L, *Bgl* II; E, *Bst*EII; H, *Hinc*II; K, *Kpn* I; S, *Sma* I; T, *Tth* I; and X, *Xma* III.

2, the 1.0-kbp fragment present in the digest of Pum$^+$ S9 DNA is replaced by a 2.1-kbp fragment in digests of SD3, SD9, SD10, SD17, and OD2 and by a 1.5-kbp fragment in digests of SD12, SD16, SD19, L33, and R1mR. The 1.0-kbp fragment formed from Pum$^+$ S9 DNA (indicated by arrow) is produced by *Xma* III cleavages, one of which occurs 200 bp upstream of the translational initiation site and the second of which occurs close to the COOH-terminal coding region of the BO gene (Fig. 1). Therefore, the Pum$^-$ mutants contain insertions of either 1.1-kbp or 500-bp DNA sequences within the BO coding region.

Pum$^-$ Strains SD3, SD9, SD10, SD17, and OD2 Contain ISH*1* at a Specific Site Within the BO Gene. We have previously shown that ISH*1*, a 1,118-bp-long transposable element, inserts into the BO gene at the same specific site in both orientations in the two Pum$^-$ mutants, pMSb1 and pSD17 (6). The following experiment was carried out to determine if the 1.1-kbp insertion in the Pum$^-$ strains SD3, SD9, SD10, and OD2 is due to the presence of ISH*1* within the BO gene. The ISH*1*-specific probe was used in Southern blot hybridization with *Xma* III digests of genomic DNAs of the above strains. As seen in Fig. 3*A*, the above four Pum$^-$ strains, as well as the previously characterized SD17 (6), all contain a 2.1-kbp fragment (indicated by arrow) that hybridizes to ISH*1*. The size of this fragment, which is absent from the digest of the wild-type Pum$^+$ strain S9, is identical to that of the *Xma* III fragment from these strains, which contains the BO gene (Fig. 2, left five lanes). Thus, the Pum$^-$ strains SD3, SD9, SD10, and OD2 contain ISH*1* inserted within the BO coding region.

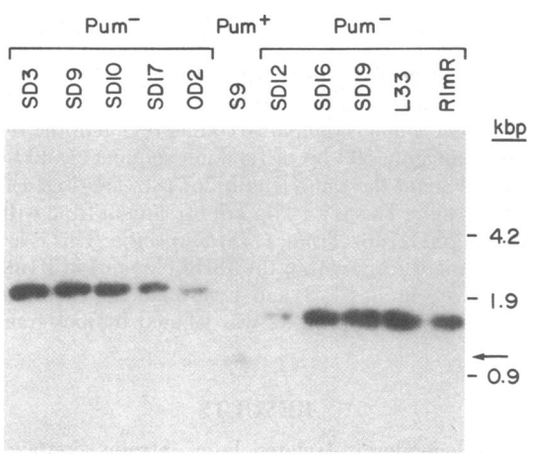

FIG. 2. Identification of the BO gene in restriction digests of Pum$^+$ and Pum$^-$ strains of *H. halobium*. Southern hybridization using BO gene-specific probe to *Xma* III digests of genomic DNA from Pum$^+$ strain S9 and Pum$^-$ strains SD3, SD9, SD10, SD17, OD2, SD12, SD16, SD19, L33, and R1mR. The arrow marks the position of the 1-kbp *Xma* III fragment that hybridizes to the probe in the digest of Pum$^+$ S9 DNA. Numbers on the side indicate DNA size markers.

The sites of insertion of ISH*1* in these Pum$^-$ strains were mapped by using the 1.6-kbp *Bam*HI/*Bst*EII fragment as the probe with *Sma* I digests of their DNAs. *Sma* I cleaves *H. halobium* DNA 450 bp upstream and ≈1.7 kbp downstream from the BO coding sequence, and it cleaves once within ISH*1* approximately 50 bp from one end (Fig. 1). Every one of the Pum$^-$ strains examined yielded two bands that hybridized to the probe (Fig. 3), supporting the conclusion that these Pum$^-$ strains all contain inserts of ISH*1* within the BO gene. The sizes of these bands for strains SD3, SD9, and OD2, 1.5 and 2.5 kbp, are identical to those seen in SD17. Thus, strains SD3, SD9, and OD2 contain ISH*1* inserts at the same site, within the limit of resolution of the gel, and in the same orientation as in the previously characterized Pum$^-$ strain SD17 (6). In contrast, the Pum$^-$ strain SD10 yielded *Sma* I fragments, 500 bp and 3.5 kbp, that hybridized to the probe. These would be the expected fragments if SD10 contains ISH*1* at exactly the same site within the BO coding sequence but in the opposite orientation, as in pMSb1 (6).

Pum$^-$ Strains SD12, SD16, SD19, L33, and R1mR All Contain an Approximately 500-bp-long Insertion Element, ISH2, Within the BO Coding Region. Evidence for this conclusion was obtained from Southern blot hybridization and from sequence analysis of the cloned DNAs from three of the five Pum$^-$ strains (see below). An ISH2-specific probe was used for hybridization to *Xma* III digests of the DNAs of the above strains. The results in Fig. 4*A* show that all five Pum$^-$ strains contain a common fragment, indicated by an arrow, that hybridizes to

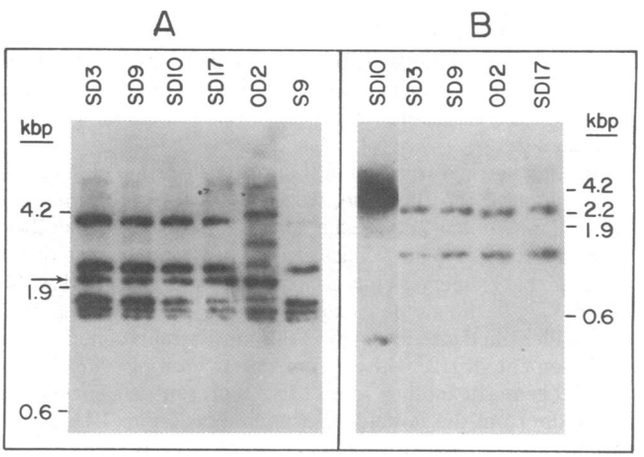

FIG. 3. Identification of Pum$^-$ *H. halobium* strains containing insertions of ISH*1* into the BO gene (*A*) and mapping of the site of the insertions (*B*). (*A*) Southern hybridization of ISH*1*-specific probe to *Xma* III digests of genomic DNA from Pum$^-$ strains SD3, SD9, SD10, SD17, and OD2 and Pum$^+$ strain S9. The arrow indicates the position of the extra bands hybridizing for the Pum$^-$ strains; these bands correspond to the insertions in the BO gene. (*B*) Southern hybridization using the 1.6-kbp *Bam*HI/*Bst*EII fragment containing the BO gene as probe to *Sma* I digests of genomic DNA from Pum$^-$ strains. The numbers on the sides are DNA size markers.

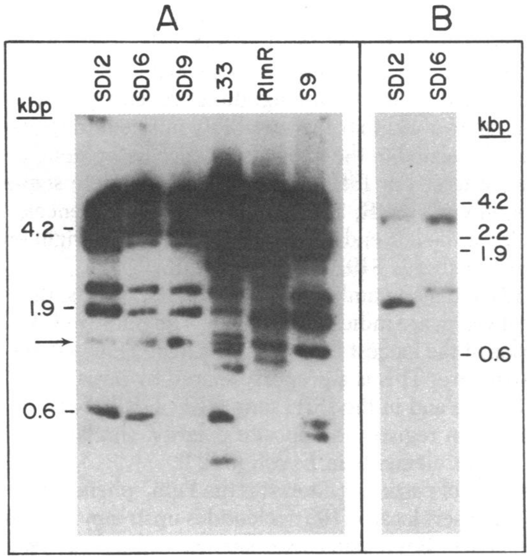

FIG. 4. Identification of Pum⁻ *H. halobium* strains containing insertions of ISH2 in the BO gene region (*A*) and mapping of the sites of the insertions (*B*). (*A*) Southern hybridization of ISH2-specific probe to *Xma* III digests of genomic DNA from Pum⁻ strains SD12, SD16, SD19, L33, and R1mR and Pum⁺ strain S9. The arrow indicates the position of the bands hybridizing for the Pum⁻ strains; these bands correspond to the insertion in the BO gene region. (*B*) Southern hybridization using the 1.6-kbp *Bam*HI/*Bst*EII fragment as probe to *Sma* I and *Bgl* II double digests of genomic DNA from Pum⁻ strains SD12 and SD16. The numbers on the sides are DNA size markers.

the ISH2 probe. (This fragment is absent from the Pum⁺ strain S9 digest.) This 1.5-kbp fragment corresponds in size to the fragment that contains the BO coding sequence in these Pum⁻ strains (Fig. 2, right five lanes).

In addition to the above fragment, several other fragments in *Xma* III digests of *H. halobium* DNA hybridize to the ISH2-specific probe (Fig. 4*A*). This indicates that *H. halobium* DNA carries multiple copies of ISH2 or elements closely related to ISH2.

ISH2 Insertion Sites Within the BO Coding Region in Pum⁻ Strains. Sites of ISH2 insertion in SD12 and SD16 were mapped as follows: A 1.6-kbp *Bam*HI/*Bst*EII fragment was used as probe with *Sma* I and *Bgl* II double digests of genomic DNA. *Sma* I cleaves Pum⁺ *H. halobium* DNA 450 bp upstream and ≈1.7 kbp downstream of the BO coding sequence (Fig. 1). The resulting *Sma* I fragment containing the BO gene is not cleaved by *Bgl* II. However, if an ISH2 element is present within the *Sma* I fragment in the Pum⁻ mutant, it will be cleaved twice by *Bgl* II within the ISH2 sequence. Results in Fig. 4*B* show two bands for both SD12 and SD16 that hybridize to the probe. (The third fragment originating from within ISH2 would not be expected to hybridize to the *Bam*HI/*Bst*EII probe used.) The presence of two hybridization bands further supports the conclusion that SD12 and SD16 contain insertions of ISH2 within the BO coding region. From the sizes of these hybridization bands and additional mapping with *Bgl* I, *Kpn* I, and *Ava* I (data not shown), we conclude that the sites of insertion and the orientation of ISH2 in SD16 and SD12 are as shown in Fig. 1. Thus, in SD16, ISH2 inserts near the COOH-terminal coding region of the BO gene downstream from the *Ava* I site, and its orientation is the same as in SD19, L33, and R1mR. On the other hand, in SD12, ISH2 inserts immediately upstream of the *Kpn* I site and has an orientation opposite to that in the above four strains (Fig. 1).

Sites of ISH2 insertion in strains SD19, L33, and R1mR were

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             -110       -100                 -100       -90        -80
SD19   TGGAATCCGCGTGTCGGCTCCGTG  ISH2  CGGCTCCGTGTCTGACGGTTCATC
       ACCTTAGGCGCACAGCCGAGGCAC        GCCGAGGCACAGACTGCCAAGTAG

        220       230                         240
L33    ATGCTGCTGGGGTATGGCCTCACA  ISH2  TGGCCTCACAATGGTAC
       TACGACGACCCCATACCGGAGTGT        ACCGGAGTGTTACCATG

           270       280                   270       280
RlmR   CAGAACCCCATCTACTGGGCGCGG  ISH2  ACCCCATCTACTGGGCGCGGTACG
       GTCTTGGGGTAGATGACCCGCGCC        TGGGGTAGATGACCCGCGCCATGC
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FIG. 5. DNA sequences flanking the insertions of ISH2 in cloned DNA for Pum⁻ strains SD19, L33, and R1mR. The ISH2 elements are flanked by either 10-bp (for SD19 and L33) or 20-bp (for R1mR) direct repeats (as indicated by boxes and arrows). The numbers above the DNA sequences refer to the position relative to the initiator codon for BO.

determined more precisely by analysis of junction sequences between the ISH2 element and flanking DNA sequences (14). For SD19, the two *Bgl* II sites within the insertion were used for determining the sequences of both junctions. For L33, the *Ava* I site was used for analyzing the left junction and the *Kpn* I site for analyzing the right junction. For R1mR, the *Kpn* I site was used for analyzing the left junction and the *Hinc*II site for the right junction (Fig. 1). Fig. 5 shows the DNA sequences that flank the ISH2 element in SD19, L33, and R1mR. The nucleotide numbers shown above each DNA sequence indicate the position of the ISH2 element relative to the initiator codon for BO. Thus, in L33 and R1mR, ISH2 inserts within 45 bp of the same site, and in the same orientation (Fig. 1). In SD19, ISH2 inserts 102 bp upstream from the initiator codon for BO.

Sequence of ISH2. The complete sequence of ISH2 has been determined for the cloned DNA from strain R1mR (Fig. 6). For ISH2 in SD19, the sequences of 40 nucleotides from the left end and 80 nucleotides from the right end were determined. For ISH2 in L33, the sequences of 40 nucleotides from the left end and 120 nucleotides from the right end were determined.

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                                                                    60
CATTCGTCTT TAGTTAAGAA ATCGCGTGAC AGCGGTAGGA TCTCTTCGCT GTGCAAGACG
GTAAGCAGAA ATCAATTCTT TAGCGCACTG TCGCCATCCT AGAGAAGCGA CACGTTCTGC
                                                                   120
CGGCTGAGAT CTCTTTTGAT AATATTTTAA CCTAAATCGA AATAAGACGA TAATCTTACC
GCCGACTCTA GAGAAAACTA TTATAAAATT GGATTTAGCT TTATTCTGCT ATTAGAATGG
                                                                   180
GTGCACCCGG TGCACGTATT TCTAAGAGCG TCTAAGACTA TGGCTCGAAC CAAAATGGGC
CACGTGGGCC ACGTGCATAA AGATTCTCGC AGATTCTGAT ACCGAGCTTG GTTTTACCCG
                                                                   240
GTCTCCATCC GAACTGAACT CGTTGATGAA CTCGATTCAC TCGTCGATGA GTGTTCAGAT
CAGAGGTAGG CTTGACTTGA GCAACTACTT GAGCTAAGTG AGCAGCTACT CACAAGTCTA
                                                                   300
CTCGGAGCAA GCCGCTCCGA GATCGTTGAA GCCATCCTCA CAGCATATTT TCAGAACGAT
GAGCCTCGTT CGGCGAGGCT CTAGCAACTT CGGTAGGAGT GTCGTATAAA AGTCTTGCTA
                                                                   360
GAAGACCAAA TCAAACAGAC GCGAGAGCTG ATTATCCGCA ACAGAAAACG CTCTAACTCG
CTTCTGGTTT AGTTTGTCTG CGCTCTCGAC TAATAGGCGT TGTCTTTTGC GAGATTGAGC
                                                                   420
TAGAGAAGTT CGTGCACCGA GTGCACGACT TCTTAGAGCG TCATTCAAAA CCACGACCTA
ATCTCTTCAA GCACGTGGCT CACGTGCTGA AGAATCTCGC AGTAAGTTTT GGTGCTGGAT
                                                                   480
GCTAAATTAA TATCGGATTG GTCTTGGGCG AATAGAAATC TTCTCTTCAC TGTTCAGCCC
CGATTTAATT ATAGCCTAAC CAGAACCCGC TTATCTTTAG AAGAGAAGTG ACAAGTCGGG
                                          520
TACGCCATGT GGCGGTTTCG CTCTTAACTA AAGACGAATG
ATGCGGTACA CCGCCAAAGC GAGAATTGAT TTCTGCTTAC
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FIG. 6. DNA sequence of an ISH2 element. The DNA sequence was derived from the cloned ISH2 insertion in the BO gene in strain R1mR. The 19-bp inverted repeats at the termini are indicated by ← and →. Three long internal palindromes are shown by arrows between the strands. Three open reading frames (ORFs) are marked by numbered arrows for GTG start codons and boxes for stop codons. ORF I is 80 codons long (top strand, nucleotides 121–360), ORF II is 64 codons long (bottom strand, nucleotides 281–90), and ORF III is 59 codons long (bottom strand, nucleotides 414–238). ORFs I and II contain in-phase ATG codons marked by *.

These were identical to the corresponding sequences for RlmR. These results support the conclusion that SD19, L33, and RlmR all contain the same element ISH2 within the BO coding region.

ISH2, which is 520 bp long, has several features common to other transposable elements. (*i*) It is capable of transposition into sites with no significant homology to itself. (*ii*) It contains a 19-bp perfect inverted repeat at its termini (← and → in Fig. 6). (*iii*) Insertion of ISH2 leads to duplication of the target site shown within the boxed region (Fig. 5). However, in contrast to most other elements that duplicate a characteristic size DNA segment, insertion of ISH2 leads to duplication of either a 10-bp sequence or a 20-bp sequence (Fig. 5). (*iv*) ISH2 contains several short ORFs. The largest of these (Fig. 6, I, top strand) is 80 codons long and two smaller ones, II and III, present in the bottom strand, are 64 and 59 codons long, respectively. All three ORFs start with GTG codons. However, ORFs I and II also have ATG codons in phase (indicated by *) close to the GTG codons.

DISCUSSION

Previously, we described the inactivation of the BO gene by insertion of a transposable element into its coding sequence in 2 Pum^- mutants (6). We have now extended this study to a total of 11 Pum^- mutants. Eight of these were spontaneous mutants and were isolated by us, whereas the other 3 were obtained from other laboratories. We have shown that, in all of the Pum^- mutants, the BO gene has been inactivated by insertion of transposable elements. ISH*1* is present in 6 Pum^- strains and 5 more carry a second element, ISH2, within the coding region of the BO gene. In one strain, insertion of ISH2 occurs 102 nucleotides upstream from the initiator codon for BO.

ISH*1*, 1,118 bp long, was shown previously to insert into a specific site at the NH_2-terminal coding region of the BO gene in both possible orientations (6). Four Pum^- strains now studied each contain a 1.1-kbp insert and, in all cases, this insert has been identified as ISH*1*. Further, the results of restriction mapping make it likely that the site of insertion of ISH*1* is the same as identified previously. Boyer and co-workers (15) have also described seven Pum^- strains, all of which contain a 1.1-kbp insert. It is likely that all these strains also contain ISH*1*, at the same site as identified by us. Thus, one of their strains, OD2, has also been studied by us and shown to contain ISH*1*. For the other six strains studied by Boyer and colleagues, the available restriction mapping data are consistent with our above conclusions. Thus, ISH*1* inserts into and inactivates the BO gene frequently and with a high degree of site specificity.

ISH2, a second element that inactivates the BO gene, is present in multiple copies in the *H. halobium* genome, and its insertion within the BO gene is clearly less site specific (Fig. 1). This element has been sequenced and characterized in detail (Figs. 4–6). It is the smallest IS element found to date, being only 520 bp long. It has the highest A+T content found thus far in *H. halobium* DNA. It is 53% A+T, compared to 42% for *H. halobium* plasmid DNA (16, 17), 40% for ISH*1* (6), 39% for the BO gene (3), and 33% for *H. halobium* chromosomal DNA (17). ISH2 contains a number of long palindromes as noted in Fig. 6.

ISH2 has features similar to ISH*1* (6) and transposable elements from other systems (18, 19). The terminal 19 bp are perfect inverted repeats. Insertion generates a duplication of the target site that flanks ISH2 as direct repeats. Interestingly, duplications generated by ISH2 insertions can be 10 or 20 bp (Fig. 5). Nearly all transposable elements duplicate a characteristic size DNA segment, which varies from one element to another. The two known exceptions in *Escherichia coli* include IS*4*, which duplicates either 11 or 12 bp (20), and a variant of IS*1*, which duplicates 8 bp instead of its usual 9 bp (21). In eukaryotes, the *Alu* family of repeated sequences in mammals is flanked by variable-sized (7- to 20-bp-long) direct repeats (22). However, it is not known whether the variability in size of duplicated sequences is related to the known sequence heterogeneity in the *Alu* sequences. For ISH2, although the complete sequence is known only in RlmR, at least the terminal sequences, 40 nucleotides at the left end and 80 nucleotides at the right end, are identical in strains S19, L33, and RlmR.

ISH2 contains a number of relatively short ORFs, the longest three of which are indicated in Fig. 6. The putative GTG initiator codon of the longest ORF (I) can be folded into a stable stem–loop structure. This is a property shared by initiator codons in the BO gene and in the ISH*1* long ORF. ORFs I and III are in codon–codon register in opposite polarity, similar to ORFs in ISH*1* and IS elements in *E. coli* (6, 23).

A finding of particular interest is the Pum^- phenotype of SD19 due to an insert located 102 nucleotides upstream from the coding sequence. Boyer and co-workers have also described Pum^- mutants that contain insertions, different from ISH*1* and ISH2, that map up to 1,200–1,500 nucleotides upstream from the BO coding region. These mutants with alterations in a large region upstream from the initiator codon promise to be useful for studies of transcription and regulation of expression of the BO gene. We have previously shown that the 5′ end of BO mRNA extends only 3 nucleotides beyond the initiator codon (3). The effect of the ISH2 insertion in SD19 could, therefore, be due to either inactivation of the promoter or a block in the processing of a longer transcript. The availability of cloned DNAs from both the wild-type and SD19 strains now makes possible *in vitro* transcriptional studies. These should help determine the location and number of the promoters in the cloned DNAs, transcription initiation sites, and whether the BO gene is part of a coordinately regulated operon (15).

The finding that all of the 11 Pum^- mutants in our case and 11 out of 12 mutants in studies of Betlach *et al.* (15) arose by insertional inactivation provides strong support for the conclusion that this is a general mechanism of inactivation of the BO gene. The frequency of spontaneous mutation for the BO gene is about 10^{-4}. Generation of a drug-resistant mutant strain of *H. halobium* occurs at a frequency of 10^{-7} (our unpublished results). Loss of gas vacuole production occurs at a higher frequency, 10^{-2} (5). The different mutation frequencies probably reflect different underlying mechanisms—e.g., point mutations, insertions of transposable elements, and other rearrangements. Differences in transposition rates and site specificity of insertion of transposable elements probably also affect mutation rates.

The Pum^- phenotype could arise by loss of BO or retinal synthesis. We find, however, that all eight Pum^- strains that we isolated have resulted from inactivation of the BO gene. If we have not, in an unknown way, biased our screening for the mutants, this result suggests that the effective target size for inactivation of the BO gene by insertion elements is larger than that for genes required for retinal synthesis. This would be somewhat unexpected because three separate reactions are thought to be required for conversion of lycopene to retinal, presumably requiring more than one gene product (24, 25). However, as pointed out above, the BO gene may be part of a large operon. In addition, there is the possibility that the BO gene contains hot spots for insertion elements.

Note Added in Proof. The junction sequences between ISH*1* and the BO gene in the strain SD10 have now been determined. They show that in this strain ISH*1* has inserted into the BO gene at exactly the same site and in the same orientation as in pMSb1. These results fur-

ther support the conclusions reached in this paper. Quantitation of purple membrane in the various Pum$^-$ mutants described in this paper has shown that, with the exception of SD19, they contain negligible amounts (less than 3%) of purple membrane compared to the amount present in S9. SD19, which has an ISH2 insert 102 nucleotides upstream of the initiator codon for BO contains 35–50% of the level of purple membrane present in S9 and is, therefore, a leaky mutant.

We thank Dr. Nancy Kleckner for helpful suggestions. This work was supported by Grants GM 17151, GM 28289, and AI 11479 from the National Institutes of Health, Grant PCM-8110992 from the National Science Foundation, and Contract NOOO14-82-K-0668 from the Office of Naval Research. S.D. is supported by a graduate fellowship from the National Science Foundation.

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