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Saturation Mutagenesis of the TATA Box and Upstream Activator Sequence in the Haloarchaeal *bop* Gene Promoter

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Degenerate oligonucleotides were used to randomize 21 bp of the 53-bp minimal *bop* promoter in three 7-bp segments, including the putative TATA box and the upstream activator sequence (UAS). The mutagenized *bop* promoter and the wild-type structural gene and transcriptional terminator were inserted into a shuttle plasmid capable of replication in the halophilic archaeon *Halobacterium* sp. strain S9. Active promoters were isolated by screening transformants of an orange (Pum$^-$ *bop*) *Halobacterium* mutant for purple (Pum$^+$ *bop*$^+$) colonies on agar plates and analyzed for *bop* mRNA and/or bacteriorhodopsin content. Sequence analysis yielded the consensus sequence 5′-tyT(T/a)Ta-3′, corresponding to the promoter TATA box element 30 to 25 bp 5′ of the transcription start site. A putative UAS, 5′-ACCcnactagTTnG-3′, located 52 to 39 bp 5′ of the transcription start site was found to be conserved in active promoters. This study provides direct evidence for the requirement of the TATA box and UAS for *bop* promoter activity.

The halophilic archaeon *Halobacterium* sp. produces the unique membrane protein bacterio-opsin, which complexes with retinal to form bacteriorhodopsin (BR). BR forms a two-dimensional crystalline lattice, called the purple membrane, in the cell membrane of *Halobacterium* species and acts as a light-driven proton pump (15). The electrochemical proton gradient generated across the membrane is used by the cells for ATP synthesis under low-oxygen, high light intensity conditions. BR synthesis has been shown to be induced by low oxygen tension and high light intensity and supports a period of phototrophic growth (21, 29, 30).

The gene for bacterio-opsin, *bop*, was one of the first archaeal genes to be cloned (3, 10), and analysis of the transcript showed it to start only two nucleotides upstream of the ATG start codon (6). Expression of the *bop* gene was detected first in mid- to late-log-phase cultures, with maximum mRNA levels occurring in the stationary phase (17, 34). Insertions in two genes divergently transcribed from *bop*, *brp*, and *bat* resulted in greatly reduced transcription of *bop*, suggesting their involvement in its regulation (Fig. 1A) (2, 18). The *bat* gene product showed similarity to the flavin adenine dinucleotide-binding region of *nifL* (34), which functions in redox sensing in nitrogen-fixing bacteria (9). The function of the *brp* gene, which encodes a hydrophobic protein, is unknown, and the Bop$^-$ phenotype of *brp* mutants may result from polar effects on *bat*. A gene downstream of *bat*, *blp*, is transcriptionally unlinked but is regulated in a manner similar to that in which *bop* is regulated (Fig. 1A) (12).

Recently, deletion analysis of the *bop* promoter showed that a 53-bp region upstream of the transcription start site is sufficient for wild-type transcriptional activity (Fig. 1B) (12, 36). Sequence analysis of this minimal promoter region revealed weak homology to the archaeal TATA box element located approximately 25 bp 5′ of the start site. Further upstream, sequence homology was noted between the *bop* promoter and the *blp* gene promoter (12). This region was referred to as the upstream activator sequence (UAS) and predicted to be involved in *bop* gene regulation (12). In addition to the TATA box and UAS, an 11-bp alternating purine-pyrimidine sequence overlapping the TATA box and centered 23 bp 5′ of the transcription start site was also identified. The region was hypothesized to undergo a change in DNA structure under conditions of low oxygen tension to a non-B-DNA form, which could explain the observed inhibition of *bop* transcription in the presence of the DNA gyrase inhibitor novobiocin (34). Direct evidence for a supercoiling-dependent structural alteration in this region was provided by showing sensitivity to osmium tetroxide and S1 nuclease (36).

In order to study the sequence requirements for transcription in more detail, we conducted saturation mutagenesis of key sequences within the minimal *bop* promoter. In this report, we provide evidence for the requirement of the TATA box and UAS for *bop* promoter activity.

MATERIALS AND METHODS

***Halobacterium* strains and culturing.** *Halobacterium* sp. strain S9, a purple-membrane (Pum) constitutive strain, and strain SD23, a Pum$^-$ derivative of S9 with an IS*H1* insertion at the 5′ end of *bop*, have already been described (34). Culturing of these *Halobacterium* strains was done at 37°C in a CM$^+$ medium containing 4.5 M NaCl and trace metals as previously described (7). Culturing for studying effects of treatment with novobiocin (Sigma, St. Louis, Mo.) was conducted as described by Yang et al. (36). Briefly, novobiocin was added to a final concentration of 0.05 μg/ml to cultures grown to an optical density at 600 nm (OD_{600}) of 0.2 (early log phase), the cultures were allowed to grow to stationary phase (OD_{600} of >1.8) in the presence of the drug, at which point the cells were harvested for purple-membrane or RNA preparations (see below).

Mutagenesis. Mutagenesis of the promoter was accomplished by PCR amplification of the cloned *bop* gene in pMS1 (10) with a mutagenic primer having degeneracies in seven positions corresponding to the region to be mutated, NB10 for the first seven nucleotides of the UAS, starting at position −52 (5′ TCGCG GATCCTAAATTCCGTCACGAGCGTNNNNNNNTGATTGGGTCGTAGA GTTA-3′); NB11 for the subsequent seven nucleotides of the UAS, from position −46 to position −40 (5′-TCGCGGATCCGTCACGAGCGTACCATAC NNNNNNNGTCGTAGAGTTACACACATATCCTC-3′); NB6 for the TATA box (5′TCGCGGATCCGCGTACCATACTGATTGGGTCGTAGNNNNNNN CACATATCCTCGTTAGG-3′); and downstream oligonucleotide NB3 (5′-GG GAATTCTACAAGACCGAGTGG-3′). The synthetic oligonucleotides were purchased from Genosys Biotechnologies, Inc., Woodlands, Tex. The PCR am-

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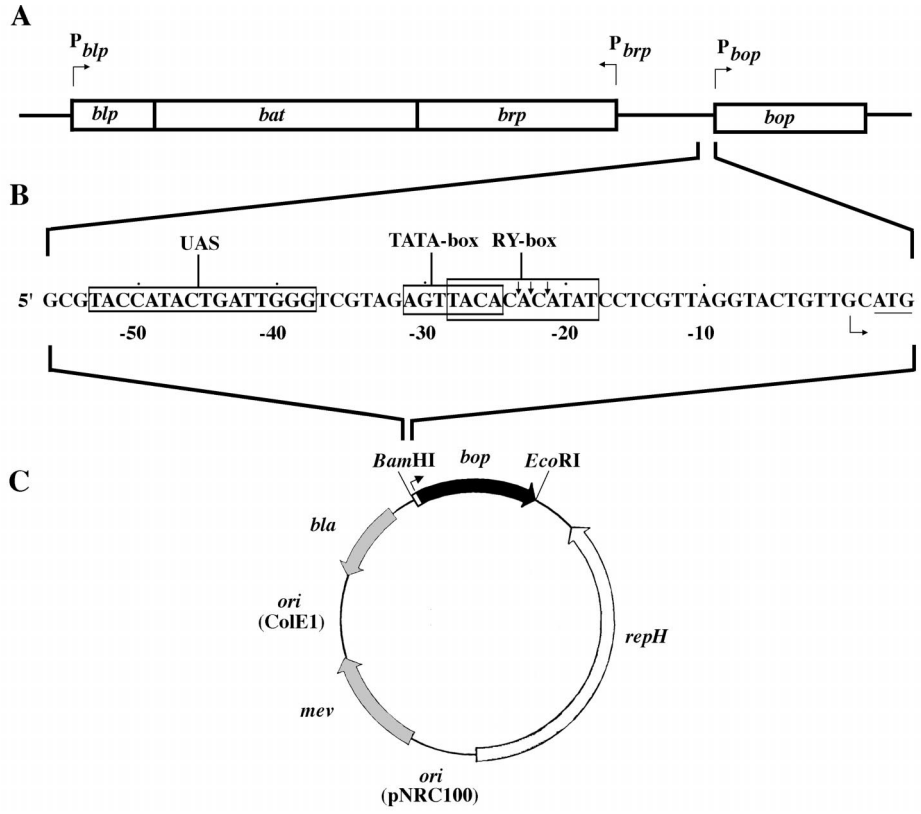

FIG. 1. Organization of the *bop* gene region and promoter mutagenesis. (A) Organization of the *bop* gene region (*bop*, *brp*, *bat*, and *blp* genes) shown in boxes with the promoters indicated above by arrows. (B) Minimum *bop* promoter and a few surrounding nucleotides. The sequence is numbered starting with the transcription start point (indicated by an arrow) as +1. The UAS, TATA box, and R-Y box regions are boxed, and the translational start codon is underlined. The locations of osmium tetroxide and S1 nuclease cleavages are indicated by vertical arrows (36). (C) *E. coli-Halobacterium* shuttle vector (pNB series), which contains the *bop* gene (black arrow) and promoter (white box). The *E. coli* replication origin (*ori* ColE1) and selectable marker (*bla* [shaded arrow]) are indicated, as are the *Halobacterium* replication origin (*ori* pNRC100), *repH* gene (white arrow), and selectable marker (*mev* [shaded arrow]).

plifications were done by using *Taq* polymerase and standard conditions on a GeneAmp PCR system 2400 thermal cycler (Perkin-Elmer, Foster City, Calif.).

Construction and screening of UAS and TATA box libraries. The products obtained from the PCR amplifications with one of the three mutagenic primers (NB10, NB11, or NB6) and NB3 were fractionated on a 0.8% agarose gel, gel purified, and digested with a fivefold excess (each) of *Eco*RI and *Bam*HI (New England Biolabs, Beverly, Mass.). The digested inserts were repurified after fractionation on a 0.8% agarose gel and used for cloning. The vector was prepared by removing a 1.2-kb *Eco*RI/*Bam*HI stuffer fragment from *Halobacterium-Escherichia coli* shuttle plasmid pNB148, a pNG168 derivative (Fig. 1C) (8), and subsequently gel purified. The insert DNAs were ligated to this vector and electroporated into Electromax *E. coli* DH10B cells (Gibco-BRL, Rockville, Md.). A fraction of the transformation was plated on Luria-Bertani agar plates containing ampicillin (100 μg/ml) to determine the efficiency of cloning, and the remainder was amplified in a 1-liter culture. Each library contained 25,000 to 30,000 members. The libraries were amplified in *E. coli* DH10B, and plasmid DNA was prepared on a large scale from a 1-liter culture by the alkaline lysis method. Plasmid DNA was purified on a CsCl density gradient and analyzed spectrophotometrically. All of the standard recombinant DNA procedures used have been previously described (27).

DNAs from the amplified libraries were transformed into Pum^- *Halobacterium* sp. strain SD23 by the polyethylene glycol-EDTA transformation method (4). *Halobacterium* transformants were selected on CM^+ plates containing 16-μg/ml mevinolin. A total of >25,000 CFU was considered a good representation of the library (each library can have a maximum of 4^7 or 16,384 different sequences). The purple-colony (Pum^+) phenotype was used as a screen to select candidates for further analysis. The presence of plasmids was confirmed by PCR analysis of total DNA from the *Halobacterium* transformants, prepared by previously described methods (19), with NB3 and T7 primers, and by recovering the plasmids after transformation into *E. coli* DH5α. Plasmid DNA extracted from single isolated *E. coli* colonies was retransformed into *Halobacterium* sp. strain SD23 to confirm the observed phenotype.

BR assays. *Halobacterium* cultures grown to an OD_{600} of approximately 1.8 were used for purple-membrane preparation by the method described by Oesterhelt (22). Cells from 50 ml of each culture were harvested by centrifugation at 7,000 rpm for 20 min. The cell paste was resuspended in 4 ml of a basal salts solution containing 20-μg/ml DNase I. The cells were gradually lysed by overnight dialysis (Spectrum Laboratories, Laguna Hills, Calif.) against distilled water, and the lysate was then analyzed spectrophotometrically at 568 nm to quantitate BR content. The absorption values were normalized to an OD_{280} of 1.5 and corrected by subtracting the background (normalized reading for host *Halobacterium* sp. strain SD23).

Primer extension analysis. Crude RNA was prepared from 3- or 6-ml *Halobacterium* cultures by either the hot-phenol method (36) or use of the RNeasy kit (Qiagen, Valencia, Calif.). Primer extension was performed on approximately 10 μg of RNA by using an end-labeled *bop*2 primer (5′CCTGCGA TACCCCCT-3′) and a primer extension kit (Promega Corporation, Madison, Wis.) in accordance with the manufacturer's instructions. End labeling was done by using [γ-^{32}P]ATP (Amersham Life Science, Arlington Heights, Ill., and NEN Life Science Products, Inc., Boston, Mass.) in a T4 polynucleotide kinase (New England Biolabs)-catalyzed reaction. The cDNA product was analyzed by electrophoresis under denaturing conditions on a 6% polyacrylamide–8.3 M urea gel. Band intensities were quantified by densitometric analysis of the autoradiograms with a Bio-Rad densitometer with Molecular Analyst software (Bio-Rad Laboratories, Hercules, Calif.) or by PhosphorImager analysis of the gel with a Storm 860 scanner connected to the PhosphorImager SI system with ImageQuant software (Molecular Dynamics, Sunnyvale, Calif.).

Sequence analysis. Promoter plasmids prepared by the alkaline lysis method from cultures of *E. coli* DH5α transformants were used as templates for sequencing. T7 and *bop*2 oligonucleotides were used to sequence both strands by the dideoxy cycle-sequencing method (25, 28) using either radioisotope-based chemistry (Genozyme Cycle-Sequencing Kit [Genomyx Corp., Foster City, Calif.] or Sequenase version 2.0 Sequencing kit [Amersham Life Science, Cleveland, Ohio]) on a Genomyx LR sequencer or fluorescent-dye terminator chemistry (Thermosequenase dye terminator cycle sequencing premix kit; Amersham Life Science) on an ABI373A sequencer (Perkin-Elmer). Sequences were analyzed by using the GCG software package (Genetics Computer Group Inc., Madison, Wis.) running on an SGI O2 workstation (Silicon Graphics Inc., Mountain View, Calif.).

RESULTS

Saturation mutagenesis of the TATA box and UAS. The *bop* gene was mutagenized by PCR amplification using one of three oligonucleotides, each with degeneracies at seven positions within the promoter sequence, and a nonmutagenic oligonucleotide located downstream of the transcriptional terminator. The mutagenic regions corresponded to two segments of the UAS and the TATA box (Fig. 1B). The amplified *bop* gene fragments were cloned into a *Halobacterium-E. coli* shuttle vector (Fig. 1C) and transformed into *E. coli*. At least 25,000 transformants were obtained for each library. Since the total number of different sequences that could be obtained with seven degeneracies was 16,384, we calculate that 78% of the possible sequences were represented. The libraries were amplified in *E. coli* DH10B and transformed into *Halobacterium* sp. strain SD23 (a *bop* mutant derivative of strain S9). Transformant colonies were visually inspected for purple-membrane content and scored as Pum^+ or Pum^-. Phenotypes were confirmed by recovery of plasmids and retransformation of SD23. Pum^+ and Pum^- representatives were analyzed for *bop* gene expression by assaying mRNA levels using primer extension analysis and/or for BR content by spectrophotometric analysis.

TATA box mutagenesis. The putative TATA box region of the *bop* promoter (31 to 25 bp 5′ of the transcription start point) was mutagenized as described above. Approximately 2 to 3% of the transformants were phenotypically Pum^+, suggesting that two or three nucleotides in this region are critical for *bop* gene expression. The levels of *bop* mRNA and BR protein produced from the wild-type promoter and four mutated Pum^+ promoters (1B2, 2B12, 2H10, and 1D2) were compared by primer extension analysis and spectrophotometric assays (Fig. 2). There was close correspondence between the Pum phenotypes, *bop* mRNA levels, and BR levels. Moreover, the transcription start points were unchanged, irrespective of the level of transcription observed.

We selected 13 Pum^+ and 4 Pum^- transformants for further characterization. The promoter regions were sequenced and aligned, and a consensus sequence was derived from the most active promoters [5′-tyT(T/a)Ta-3′, −30 to −25 bp 5′ of the transcription start point] (Fig. 3). Significantly, two positions of the consensus (two T nucleotides at positions −28 and −26) were highly conserved (present in 9 of the 10 most active promoters). A third T nucleotide (at position −27) was nearly as highly conserved, although an A is also tolerated. Those promoters containing a TTT or TAT sequence in the region between −28 and −26 (i.e., 1B2, 2H1, 2B2, 2B12, and ZA1) produced higher levels of transcription than the wild type, which contained a TAC sequence in this region. Inactive promoters (1A1, ZF6, ZF8, and 2D6), in contrast, contained two or more differences within the most highly conserved region in the consensus. All of the functional promoters were found to be inhibited or inactive in the presence of novobiocin, indicating that they are sensitive to DNA supercoiling, like the wild type.

UAS mutagenesis. Because of the larger size of the UAS region, it was mutagenized in two separate 7-bp segments, from −52 to −46 (UAS10) and from −45 to −39 (UAS11) from the transcription start site. The importance of this entire region for *bop* gene expression was shown by the finding of about 1% Pum^+ transformants, which indicated that the nucleotide sequence is somewhat less mutable than the TATA box region for active promoter activity. We selected 6 Pum^+ and 3 Pum^- transformants from the UAS10 region and 10 Pum^+ and 1 Pum^- transformants from the UAS11 region for further characterization. The BR contents in eight Pum^+ and four Pum^- mutants were analyzed spectrophotometrically (Fig. 4), and the mRNA levels in the Pum^+ mutants were measured by primer extension analysis (Fig. 5). The results confirmed the observed phenotypes. The transcription start site was found to be unchanged, irrespective of the promoter strength (Fig. 5). The consensus sequence obtained from alignment of the mutated UASs was 5′-ACCcnactagTTnG-3′, very similar (three differences in 12 bp) to the wild-type sequence, 5′-ACCATACTGATTGG-3′. Significantly, four positions (−52, −51, −41, and −39) were completely invariant within the UASs of active promoters. One mutant promoter (11-1), with improved similarity to the consensus UAS, produced 68% more BR protein than the wild type, while another (10-3) with comparable similarity produced 33% more BR than the wild type. By comparison, the inactive promoters contained multiple differences in the most-conserved nucleotides of the consensus sequence. All of the active promoters were also inhibited by novobiocin, indicating that they are sensitive to DNA supercoiling.

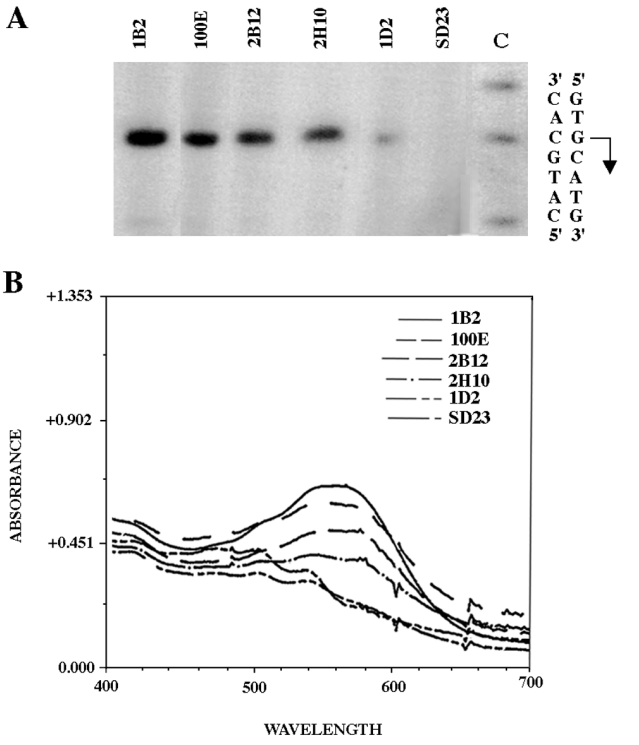

FIG. 2. Analysis of TATA box mutants at the transcriptional (A) and translational (B) levels. (A) Primer extension analysis of *bop* mRNA in four TATA box mutants using crude RNA with the *bop*2 primer. Strain designations are above the lanes. The controls used in this experiment were Pum^- (*bop*) host strain SD23, strain 100E (SD23 containing a plasmid with an unmutated promoter), and TATA box mutants (1B2, 2B12, 2H10, and 1D2). A sequencing reaction (lane C) performed with the *bop*2 primer on a pMS1 template (plasmid containing the cloned *bop* gene) is shown, as is the double-stranded sequence across the transcription start point (the start site and direction are indicated by the arrow). (B) Spectra (absorbance versus wavelength from 400 to 700 nm) of purple membrane preparations for the four TATA box mutant strains and the two control strains (same as in panel A). Relative BR content was quantified by comparison of absorbance at 568 nm.

DISCUSSION

We have conducted saturation mutagenesis of the TATA box and UAS region in the *bop* promoter. Degenerate oligonucleotides were used to produce over 25,000 different promoter mutants, which were screened by using the purple

A

Strain	-31	-30	-29	-28	-27	-26	-25	Pum	[Bacteriorhodopsin] Novobiocin -	+	Ratio
S9	A	G	T	T	A	C	A	++	1.00	0.17	0.17
100E	A	G	T	T	A	C	A	++	1.08	0.18	0.17
1B2	G	T	.	.	T	T	.	++	1.21	0.12	0.10
2H1	G	C	.	.	T	T	C	++	1.18	N D	-
2B2	.	T	C	.	T	T	.	++	1.08	N D	-
2B12	A	T	A	.	T	T	C	++	1.02	N D	-
ZA1	T	A	A	.	.	T	G	++	1.37	0.22	0.16
1A7	A	A	C	.	T	T	.	++	*	*	-
1B11	T	C	C	.	T	T	.	++	*	*	-
1H7	G	T	.	.	T	T	T	++	*	*	-
2A3	C	T	C	.	.	A	.	++	*	*	-
2H10	.	T	C	.	T	T	C	++	*	*	-
1D1	G	C	.	.	G	A	.	+	0.39	ND	-
1D2	G	A	C	.	T	A	.	+	0.50	ND	-
1H10	C	C	.	A	T	T	C	+	*	*	-
1A1	C	G	.	G	G	T	T	-	*	*	-
2D6	T	A	A	A	C	A	.	-	*	*	-
ZF6	.	T	G	G	C	G	G	-	*	*	-
ZF8	T	G	G	.	G	T	.	-	*	*	-

B

	-31	-30	-29	-28	-27	-26	-25
G	5	1	0	0	1	0	2
A	5	3	2	1	3	3	8
T	2	6	6	13	10	10	1
C	2	4	6	0	0	1	3
	n	t	y	T	T/a	T	a

FIG. 3. Tabulation of strain designations, promoter sequences, phenotypes, and BR contents of TATA box mutants (A) and analysis of promoter sequences (B). In panel A, the strain designations are shown in the first column, and the sequence of nucleotides −31 to −25 (identity to the wild type base is denoted by a dot), the Pum phenotype (−, negative; +, positive; ++, overproducer), and relative BR content are shown to the right. ND, not detectable; *, not done. For panel B, the TATA box consensus sequence was determined by tallying individual nucleotides observed at each position in Pum$^+$ strains. The consensus sequence is indicated at the bottom.

(Pum$^+$) or orange (Pum$^-$) phenotype. A wide range of phenotypes, from purple-membrane overproducers to completely purple-membrane-deficient strains, were characterized at both the transcriptional and translational levels. The results were found to be consistent at the phenotypic, mRNA, and protein levels, confirming that the observed effects resulted from promoter mutations. The mutations had no effect on the transcription start site, ruling out the possible activation of alternate promoters. Taken together, the findings constitute a detailed mutagenic analysis of two putative *bop* promoter elements with clear demonstration of their requirement for wild-type promoter activity.

The *bop* promoter has been of considerable interest because of its complex regulation (responsive to oxygen, light, and DNA supercoiling). Moreover, unlike most archaeal (and eukaryotic) promoters which have a distinctive TATA box (also called BoxA) centered at 25 bp 5′ of the transcription start site, which is recognized by the TATA-binding protein (TBP) transcription factor (1a, 11, 14, 16, 32), the *bop* promoter has weak homology to the TATA sequence located several nucleotides further upstream. Our mutagenic analysis has definitively established that the sequence in the TATA box region is involved in *bop* promoter activity. Interestingly, the wild-type *bop* promoter sequence is different from the consensus TATA box sequence, even in the most highly conserved region, and the 3′ four nucleotides of the consensus are more similar in sequence and position to the consensus sequences derived from alignment and mutagenesis of other archaeal promoters (5, 13, 23, 31).

The *Halobacterium* genome project has shown the presence of multiple TBP-encoding genes (20). Four *tbp* genes are present on a 191-kb minichromosome named pNRC100. This finding suggests that alternate TBPs are probably involved in

A

	UAS Sequence															[Bacteriorhodopsin] Novobiocin		
Strain	-52	-51	-50	-49	-48	-47	-46	-45	-44	-43	-42	-41	-40	-39	Pum	-	+	Ratio
100E	A	C	C	A	T	A	C	T	G	A	T	T	G	G	++	1.00	0.22	0.22
10-1	.	.	A	C	C	.	.	.	.	.	.	.	.	.	+	0.23	*	*
10-2	.	.	.	C	C	T	.	.	.	.	.	.	.	.	++	0.80	0.02	0.02
10-3	.	.	.	G	A	.	.	.	.	.	.	.	.	.	++	1.32	0.15	0.12
10-7	.	.	.	C	.	.	T	.	.	.	.	.	.	.	++	0.82	0.11	0.13
10-8	.	.	.	C	A	G	A	.	.	.	.	.	.	.	+	*	*	-
10-11	.	.	.	C	.	T	T	.	.	.	.	.	.	.	+	*	*	-
10-4	C	T	G	T	G	G	T	.	.	.	.	.	.	.	-	ND	ND	-
10-5	C	G	G	C	C	T	G	.	.	.	.	.	.	.	-	ND	ND	-
10-9	T	G	T	C	C	T	T	.	.	.	.	.	.	.	-	ND	ND	-
11-1	.	.	.	.	.	.	.	.	A	.	.	.	T	.	+++	1.68	0.26	0.15
11-2	.	.	.	.	.	.	.	C	A	C	.	.	T	.	++	0.61	0.16	0.26
11-5	.	.	.	.	.	.	.	.	A	.	.	.	T	.	++	*	*	-
11-6	.	.	.	.	.	.	.	.	A	G	C	.	T	.	++	0.66	0.13	0.20
11-7	.	.	.	.	.	.	.	A	A	G	.	.	C	.	++	*	*	-
11-8	.	.	.	.	.	.	.	.	A	.	.	.	T	.	++	*	*	-
11-10	.	.	.	.	.	.	.	A	.	G	.	.	.	.	++	0.75	0.12	0.16
11-11	.	.	.	.	.	.	.	.	A	G	.	.	A	.	++	*	*	-
11-14	.	.	.	.	.	.	.	.	A	T	.	.	C	.	++	*	*	-
11-15	.	.	.	.	.	.	.	.	A	G	.	.	.	.	++	*	*	-
11-12	.	.	.	.	.	.	.	C	C	.	A	.	T	T	-	ND	ND	-

B

	-52	-51	-50	-49	-48	-47	-46	-45	-44	-43	-42	-41	-40	-39
G	0	0	0	1	0	1	0	0	2	6	0	0	3	11
A	7	0	1	1	2	4	1	3	9	3	0	0	1	0
T	0	0	0	0	2	2	2	7	0	1	10	11	5	0
C	0	7	6	5	3	0	4	1	0	1	1	0	2	0
	A	C	C	c	n	a	c	t	a	g	T	T	n	G

FIG. 4. Tabulation of strain designations, promoter sequences, phenotypes, and BR contents of UAS mutants (A) and analysis of promoter sequences (B). In panel A, the strain designations are shown in the first column, and sequence of nucleotides −52 to −39 (identity to the wild type base is denoted by a dot), the Pum phenotype (for definitions, see the legend to Fig. 3), and relative BR content are shown to the right. ND, not detectable; *, not done. For panel B, the UAS element consensus sequence was determined by tallying individual nucleotides observed at each position in Pum^+ strains. The consensus sequence is indicated at the bottom.

the recognition of different subsets of genes in the *Halobacterium* genome. If so, it is possible that an alternate TBP is involved in the recognition of the *bop* promoter in the wild type. A similar mechanism may be used to regulate transcription of heat shock promoters in the related halophile *Haloferax volcanii* (33). It is also possible that different TBP factors are involved in promoting the transcription of some mutated and wild-type *bop* promoters. However, the same transcription start site is used in all cases.

The UAS has been hypothesized to function in *bop* gene regulation (12, 36). Deletion analysis showed that the UAS is required for *bop* transcription, and the sequence requirement in this region has been confirmed by the mutagenic analysis described in this report. The consensus sequence of the UAS derived from mutagenesis has several interesting properties, including a sequence slightly different from that of the wild type, which likely explains the less-than-maximum promoter activity observed in the wild type. A greater degree of conservation is observed near the 5′ and 3′ ends than in the middle. It is likely that the UAS is a site of action of a global regulator of gene expression, which is suggested by its occurrence at many genomic sites (1). An interesting possibility is that the *bop* regulatory gene products, BRP and/or BAT (or a protein interacting with these proteins), bind to the UASs near the *bop*, *blp*, and other genes and modulate transcription from these promoters in response to oxygen and/or light.

One of the most intriguing aspects of *bop* promoter function is its property of supercoiling sensitivity. We previously observed that like that of some other supercoiling sensitive genes in bacteria (24), the DNA gyrase inhibitor reduces *bop* transcription by a factor of 5 to 10 at concentrations subinhibitory for growth (35). Novobiocin prevents the increased supercoiling observed at late logarithmic phase which accompanies *bop* gene induction. At the high negative supercoiling density found under inducing conditions, the 11-bp alternating purine-pyrimidine sequence (the R-Y box) adopts a non-B-DNA structure. We hypothesized that the supercoiling-stimulated non-B-DNA structure is necessary for full induction of the *bop* gene via transcriptional activation. Mutagenesis of the UAS and TATA box showed that neither region is responsible for the sensitivity of the promoter to supercoiling. Preliminary data suggest that the region 3′ of the TATA box (middle of the alternating purine-pyrimidine R-Y box region) mediates the response to a change in supercoiling (1).

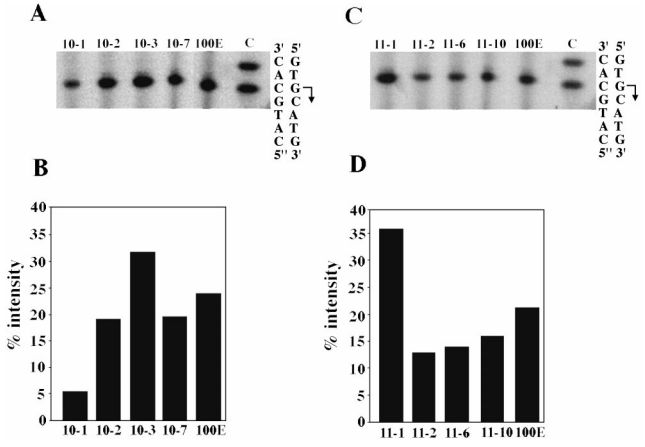

FIG. 5. Effect of UAS mutagenesis on transcription start site location (A and C) and promoter strength (B and D). For panels A and B, transcription start sites were mapped by comparing the mobilities of primer extension products to that of a sequencing ladder generated with the *bop*2 primer and the pMS1 template (plasmid with a cloned *bop* gene). Lane C of the sequencing reaction is shown. The strain designations are shown over the corresponding lanes. For panels C and D, promoter strength was measured by quantifying the *bop* message by PhosphorImager analysis of the primer extension gels. The strain designations (corresponding to A and C, respectively) are shown below the bars plotted against relative intensity.

The results obtained thus far have established the importance of the TATA box and the UAS in *bop* gene expression and suggested the involvement of transcription factors such as a TBP and other regulatory proteins in the activation and modulation of transcription. However, more detailed understanding of the mechanisms of promoter recognition, activation, and regulation requires further genetic and biochemical analysis, including development of an in vitro transcription system using both purified proteins and DNA topoisomers.

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ADDENDUM IN PROOF

Further mutagenesis has shown the requirement of a G at position −38, which is likely to be part of the functional UAS.

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