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Genetic and Topological Analyses of the *bop* Promoter of *Halobacterium halobium*: Stimulation by DNA Supercoiling and Non-B-DNA Structure

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The *bop* gene of wild-type *Halobacterium halobium* NRC-1 is transcriptionally induced more than 20-fold under microaerobic conditions. *bop* transcription is inhibited by novobiocin, a DNA gyrase inhibitor, at concentrations subinhibitory for growth. The exposure of NRC-1 cultures to novobiocin concentrations inhibiting *bop* transcription was found to partially relax plasmid DNA supercoiling, indicating the requirement of high DNA supercoiling for *bop* transcription. Next, the *bop* promoter region was cloned on an *H. halobium* plasmid vector and introduced into NRC-1 and S9, a *bop* overproducer strain. The cloned promoter was active in both *H. halobium* strains, but at a higher level in the overproducer than in the wild type. Transcription from the *bop* promoter on the plasmid was found to be inhibited by novobiocin to a similar extent as was transcription from the chromosome. When the cloned promoter was introduced into S9 mutant strains with insertions in either of two putative regulatory genes, *brp* and *bat*, no transcription was detectable, indicating that these genes serve to activate transcription from the *bop* promoter in *trans*. Deletion analysis of the cloned *bop* promoter from a site ~480 bp upstream of *bop* showed that a 53-bp region 5′ to the transcription start site is sufficient for transcription, but a 28-bp region is not. An 11-bp alternating purine-pyrimidine sequence within the functional promoter region, centered 23 bp 5′ to the transcription start point, was found to display DNA supercoiling-dependent sensitivity to S1 nuclease and OsO_4, which is consistent with a non-B-DNA conformation similar to that of left-handed Z-DNA and suggests the involvement of unusual DNA structure in supercoiling-stimulated *bop* gene transcription.

The *bop* gene, encoding bacterio-opsin, the purple membrane protein of *Halobacterium halobium*, was one of the first archaeal genes to be cloned and sequenced (11). After cloning, several studies focused on the transcription of the *bop* gene and its regulation by oxygen and light. The start site for transcription was shown to map just 2 nucleotides upstream of the coding region by purification of the message, capping of its 5′ end, and sequencing (6). Although this established *bop* mRNA as a primary transcript, no easily identifiable promoter could be found. Only a weak similarity to the TATA-like (box A) element was present (17). Interestingly, an 11-bp-long alternating purine-pyrimidine sequence centered 23 bp 5′ to the transcription start site was observed and hypothesized to influence transcription by adopting left-handed Z-DNA conformation, which would be stabilized by high salt concentration and DNA supercoiling in *H. halobium.*

The *bop* gene was shown to be transcriptionally induced at least 20-fold under microaerobic conditions in wild-type *H. halobium* strains (36, 43). Interestingly, *bop* gene transcription was inhibited by novobiocin, suggesting the involvement of DNA supercoiling in the modulation of promoter activity (42). Moreover, the involvement of two upstream genes in *bop* gene transcription was revealed by analysis of purple membrane-deficient (Pum^-) mutants of *H. halobium* (1, 25). These two genes, *brp* and *bat*, are organized like an operon with a divergent transcriptional orientation to *bop* (Fig. 1). Genetic and sequence analyses suggested that the *bat* gene product is likely to function in sensing oxygen (14, 43), but the function of the *brp* gene product, a hydrophobic protein, is unknown. Mutants overexpressing purple membrane (e.g., S9) have also been isolated, but the genetic basis for this phenotype is not known.

In this work, we have sought to better understand the complex interrelationships among DNA supercoiling, DNA structure, and *brp* and *bat* gene products in the regulation of *bop* promoter activity in *H. halobium.*

MATERIALS AND METHODS

***H. halobium* strains and culturing.** *H. halobium* NRC-1 is a wild-type strain, and S9 is a purple membrane overproducer strain, both of which were previously described (35, 39). Strains SD20, SD23, and SD27, purple membrane-deficient mutants of S9, were recently isolated (42). *H. halobium* was cultured in a medium containing 4.5 M NaCl at 37°C with illumination (6). For treatment with novobiocin (Sigma, St. Louis, Mo.), cultures were grown to an optical density at 600 nm (OD_{600}) of 0.2 prior to the addition of this drug.

RNA purification and quantitation. For RNA preparation, cultures at the appropriate growth stage were chilled at 4°C, centrifuged at 8,000 × *g* for 10 min, lysed by the addition of 1/10 volume of TE (10 mM Tris-HCl [pH 7.5], 1 mM EDTA), and extracted three times with an equal volume of phenol prewarmed to 65°C and twice with chloroform. RNA was precipitated with 2.5 volumes of −20°C ethanol after the addition of sodium acetate to 0.3 M, centrifuged at 10,000 × *g* for 10 min, and dissolved in TE. RNA was quantified by measuring A_{260} and by staining with ethidium bromide after agarose gel electrophoresis.

RNA quantification by primer extension analysis was carried out on 20 μg of crude RNA. Oligonucleotide primers were first 5′ end labeled with T4 polynucleotide kinase (Bethesda Research Laboratories, Gaithersburg, Md.) and [γ-^{32}P]ATP (Amersham, Arlington Heights, Ill.) and extended with avian myeloblastosis virus reverse transcriptase (Promega, Madison, Wis.), as previously described (43). The sequences of the oligonucleotides were 5′-CCTGCGATAC CCCCT-3′ for the *bop* transcript from the chromosome or the pLM series in the SD23 background, 5′-AGCGGATAACAATTTCACACAGG-3′ for the *bop* transcript from the pLM series in S9, and 5′-TGGGATGTCGATGAG-3′ for the *bop* transcript from plasmid pCY33. Reaction products were analyzed on gels of 8% polyacrylamide–8.3 M urea or 20% polyacrylamide—7 M urea, with subsequent autoradiography. All experiments were carried out in duplicate or triplicate with RNA isolated from independent cultures. Bands were quantitated by densitometric analysis of X-ray films using the PDI (Huntington Station, N.Y.) densitometer and Quantity One software running on a Sun Sparcstation IPC workstation (Mountain View, Calif.).

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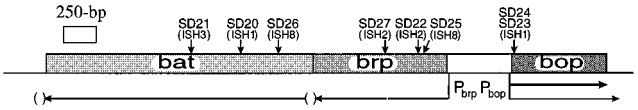

FIG. 1. Structure and mutation of the *bop* gene region in *H. halobium*. The coding regions for three genes, *bop*, *brp*, and *bat*, are indicated by shaded boxes. The locations of insertion elements in Pum$^-$ mutants are shown by vertical arrows, and their identities are indicated parenthetically with strain names shown above (42). The divergent promoters between *bop* and *brp* are labeled P_{bop} and P_{brp}, respectively, and the directions and extents of transcription are indicated by horizontal arrows below coding regions. Two species of *bop* mRNAs, major and minor, are distinguished by heavy and light arrows, respectively. Uncertainties about the termini for *brp* and *bat* transcripts are indicated by parentheses.

***H. halobium* plasmids and transformation.** Plasmid pNG11 (15.5 kb), which contains the origin of replication of the 200-kb *H. halobium* NRC-1 plasmid pNRC100, the *Haloferax volcanii mev* gene (22), and the pTZ19r *Escherichia coli* plasmid (Pharmacia-LKB, Piscataway, N.J.), has been previously described (28). Plasmid pCY1 was constructed by the ligation of two fragments, a 3.5-kb *Bam*HI fragment containing the *mev* gene from pNGMEV100 (28) and the *Sal*I fragment of 1.8-kb *Halobacterium* plasmid pGRB1 (16). Plasmid pCY33 (5.9 kb) was constructed by inserting into pCY1 by a multistep procedure a 601-bp fragment containing the *bop* promoter which had previously been amplified by PCR (32). For PCR amplification, two oligonucleotides, 5′-CCTGCGATACCCCCT-3′ and 5′-CACGACGGGACGAAC-3′, hybridizing near the 5′ ends of the *bop* and *brp* genes, respectively, were used as primers, and pMS1, which contains the cloned S9 *bop* gene (11), was used as the template. Plasmid pJKbop was constructed by inserting an 810-bp *Ava*II fragment containing the entire *bop-brp* intergenic region and the 5′ ends of the coding regions into the filled-in *Eco*RI sites of 6.3-kb *E. coli-H. halobium* shuttle plasmids containing the *mev* gene, pGRB1, and pUC12. The pLM plasmid series was generated from pJKbop by digestion with *Bst*XII and *Bbs*I, followed by the exonuclease III-mung bean nuclease nested deletion procedure according to the manufacturer's instructions (Stratagene, La Jolla, Calif.). Recombinant DNA procedures were carried out as described by Sambrook et al. (33). The construction of pCY1 and pCY33 was carried out in *H. halobium* NRC-1. The transformation of *H. halobium* was done by the polyethylene glycol-EDTA procedure of Cline and Doolittle (5). Transformants were selected by plating on agar plates containing 10 μg of mevinolin per ml. Mevinolin was added at the same concentration for growth in liquid culture, and plasmids were prepared by a previously described method (29).

Structural analysis of the *bop* promoter region. The *bop* promoter region of pCY33 was analyzed for non-B-DNA structure with OsO_4 and S1 nuclease (21). For OsO_4 treatment, 0.1 μg of pCY33 DNA per ml was incubated in a solution containing 0.1 M NaCl, 25 mM Tris-HCl (pH 7.5), 2 mM EDTA, 2 mM OsO_4, and 2 mM bipyridine at 37°C for 10 min. The reaction mixture was passed through a 1-ml Sephadex G-50 spin column (at 800 rpm in a Sorvall GLC centrifuge), and DNA was concentrated by ethanol precipitation. For S1 nuclease assays, 0.1 μg of pCY33 DNA per ml was treated with 0.5 U of S1 nuclease (Sigma) in a 30-μl reaction mixture containing 50 mM sodium acetate (pH 4.6), 50 mM NaCl, and 1 mM zinc acetate at 37°C for 10 min. The reaction mixture was extracted twice with phenol and once with chloroform and then concentrated by ethanol precipitation. OsO_4- and S1-treated DNA was denatured by treatment with 0.4 M NaOH at room temperature for 10 min and then neutralized by the addition of sodium acetate (pH 5.2) to 0.45 M and subsequent ethanol precipitation. Primer extension was carried out at 37°C for 1 h in 12-μl reaction mixtures containing 0.5 pmol of primer labeled at the 5′ end with T4 polynucleotide kinase and [γ-^{32}P]ATP (either the *bop* primer described above or a reverse *bop* primer, 5′-CGCTCCGTGTCTGACG-3′), 40 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, 50 mM NaCl, 0.05 mM (each) deoxynucleoside triphosphates, and 0.2 U of Klenow fragment of DNA polymerase I. The reaction products were electrophoresed on an 8% polyacrylamide–8.3 M urea gel.

DNA sequencing. DNA sequencing was carried out by the Sanger chain termination method (34) on plasmid DNA templates and synthetic oligonucleotide primers by using the U.S. Biochemicals (Cleveland, Ohio) sequencing kit containing Sequenase version 2.0.

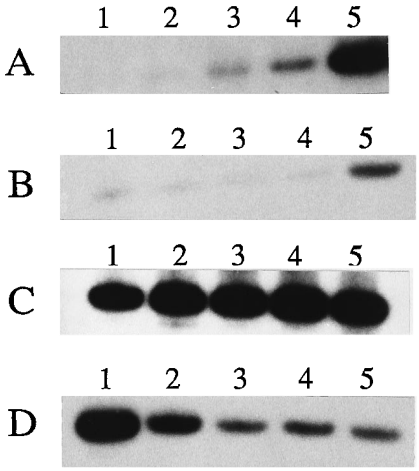

FIG. 2. *bop* message levels in *H. halobium* NRC-1 and S9 at various growth stages and after the addition of novobiocin. Message levels were assayed by primer extension analysis. (A) mRNA level in NRC-1 at an OD_{600} of 0.3 (lane 1), 0.6 (lane 2), 1.3 (lane 3), 2.3 (lane 4), and 2.5 (lane 5). (B) mRNA level in NRC-1 grown to an OD_{600} of 1.8 with novobiocin at 0.1 (lane 1), 0.5 (lane 2), 1.0 (lane 3), or 1.5 (lane 4) μg/ml or in the absence of this drug (lane 5). (C) mRNA level in S9 at an OD_{600} of 0.2 (lane 1), 0.4 (lane 2), 1.0 (lane 3), 1.3 (lane 4), and 2.3 (lane 5). (D) mRNA level in S9 grown to an OD_{600} of 1.8 in the absence of novobiocin (lane 1) or in the presence of novobiocin at 0.1 (lane 2), 0.5 (lane 3), 1.0 (lane 4), or 1.5 (lane 5) μg/ml.

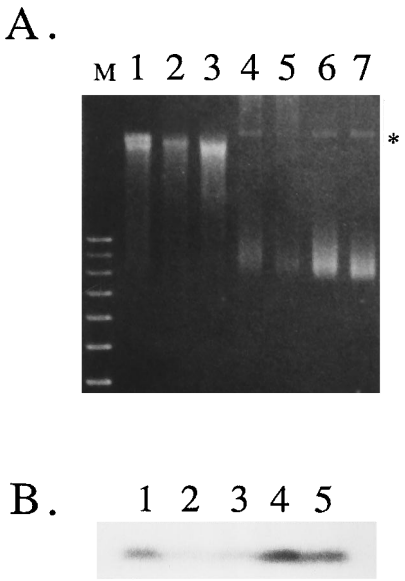

FIG. 3. Effects of novobiocin concentrations on DNA supercoiling (A) and *bop* mRNA levels (B) in *H. halobium* NRC-1. (A) Migration on an ethidium bromide-agarose gel of reporter plasmid pNG11 isolated from either *E. coli* DH5α (lane 1) or *H. halobium* NRC-1 grown to stationary phase in the presence of novobiocin at a concentration of 0.1 (lane 2), 0.05 (lane 3), 0.01 (lane 4), or 0.005 (lane 5) μg/ml. Also included was plasmid pNG11 isolated from NRC-1 cultures grown to early logarithmic phase (OD_{600} = 0.5) (lane 6) or stationary phase (OD_{600} = 2.5) (lane 7) in the absence of novobiocin. Lane M contains kilobase ladders (Bethesda Research Laboratories). Electrophoresis was carried out on a 0.5% agarose gel containing 0.02 μg of ethidium bromide per ml for 14 h. Plasmids were relaxed (lanes 1 to 3) or negatively supercoiled (lanes 4 to 7) under these electrophoretic conditions. The asterisk indicates the migration position of nicked-form DNA. (B) Primer extension analysis using the *bop* oligodeoxyribonucleotide as primer on RNA isolated from strain NRC-1 treated with novobiocin at a concentration of 0 (lane 1), 0.1 (lane 2), 0.05 (lane 3), 0.01 (lane 4), or 0.005 (lane 5) μg/ml. cDNA products were electrophoresed on a 20% polyacrylamide–7 M urea gel.

RESULTS

Transcription of the *bop* gene in *H. halobium* NRC-1 and S9. The *bop* message abundances in wild-type *H. halobium* NRC-1 and purple membrane overproducer strain S9 were assayed by primer extension analysis. Initially, RNA was isolated from batch cultures grown to various ODs in the absence of novobiocin, and an oligonucleotide specific for *bop* mRNA was used to prime the synthesis of a 45-nucleotide cDNA, consistent

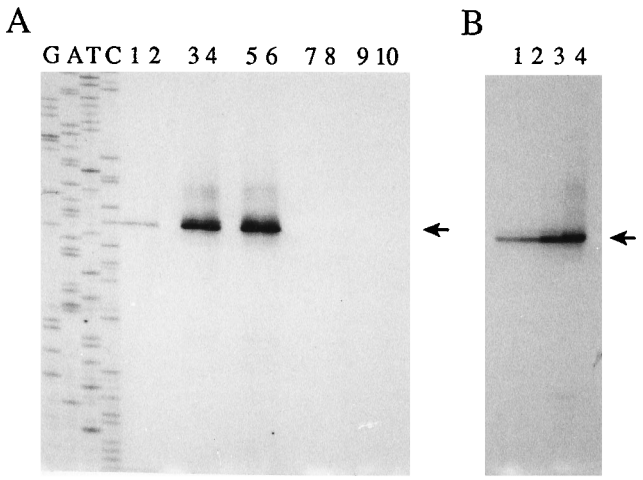

FIG. 4. Activity of the *bop* promoter located on *H. halobium* plasmid pCY33. (A) The *bop* message levels were determined in the following *H. halobium* strains transformed with pCY33: NRC-1 (lanes 1 and 2), S9 (lanes 3 and 4), SD23 (S9 *bop*::IS*H1*) (lanes 5 and 6), SD27 (S9 *brp*::IS*H2*) (lanes 7 and 8), and SD20 (S9 *bat*::IS*H1*) (lanes 9 and 10). The promoter activity of *bop* was measured by primer extension analysis of RNA from these strains with the oligonucleotide hybridizing to vector sequences as the primer and by electrophoresis on an 8% polyacrylamide–8.3 M urea gel. Lanes G, A, T, and C contained DNA sequencing ladders generated by using pCY33 as the template and the same 5′-end-labeled oligonucleotide as the primer. (B) Effect of novobiocin on the plasmid-derived *bop* promoter activity in SD23(pCY33) cultures treated with novobiocin at a concentration of 0.1 (lane 1), 0.05 (lane 2), 0.005 (lane 3), or 0 (lane 4) μg/ml. The position of *bop* message is indicated by an arrow to the right of each panel.

with the previously mapped start sites for transcription (6). The quantity of cDNA produced was shown to be proportional to the mRNA abundance when normalized to total RNA in the reaction mixture (data not shown). For the wild-type NRC-1 strain, the *bop* mRNA level increased about 20-fold (Fig. 2A) from mid-growth stage to late growth stage, confirming previous results (43), while for the purple membrane overproducer strain S9, mRNA was found at equally high levels at all growth stages (Fig. 2C), at slightly higher amounts than the induced levels in NRC-1.

Effects of novobiocin on transcription and DNA supercoiling. The induction of *bop* transcription in NRC-1 was previously shown to be blocked by the DNA gyrase inhibitor novobiocin (43). To determine the effect of this drug on high-level *bop* transcription in strain S9, RNA was isolated from cultures grown for three to four generations in the presence of 0.1 to 1.5 μg of novobiocin per ml and subjected to primer extension analysis, as described above. At these concentrations of novobiocin, cultures grew at slightly (up to 25%) reduced rates but reached stationary-phase ODs similar to those of control cultures. The levels of *bop* transcripts in both NRC-1 and S9 were found to be substantially lower at these concentrations of novobiocin (Fig. 2B and D).

Next, we compared the effect of novobiocin on the DNA supercoiling of a 15.5-kb *E. coli-H. halobium* shuttle plasmid, pNG11 (28), by ethidium bromide-agarose gel electrophoresis (Fig. 3A) with its effect on *bop* mRNA levels as measured by primer extension analysis (Fig. 3B). Both plasmid DNA and total RNA were isolated from *H. halobium* NRC-1 cultures grown for three to four generations in novobiocin from 0 to 0.1 μg/ml, and the same plasmid was also isolated from *E. coli* grown in the absence of novobiocin. The concentration of ethidium bromide used on the agarose gel (Fig. 3A) was sufficient to completely relax the plasmid DNA isolated from *E. coli* but not that from *H. halobium* in the absence of novobiocin. A comparison of the results in Fig. 3A and B shows that 0.1 or 0.05 μg of novobiocin per ml (lanes 2 and 3) inhibited the accumulation of *bop* mRNA and also substantially reduced the linking deficiency of pNG11, compared with the results for cultures treated with 0.01 μg/ml (lanes 4), 0.005 μg/ml (lanes 5), or no drug (Fig. 3A, lanes 6 and 7, and B, lane 1). The plasmid linking deficiency of pNG11 in cultures treated with 0.1 or 0.05 μg of novobiocin per ml appeared to be similar to that of the same plasmid in *E. coli* (Fig. 3A, lanes 1 through 3 [about 30% less than in the absence of novobiocin] [data not shown]). A slightly lower linking deficiency for pNG11 (much less than with novobiocin) was observed with early-log-phase

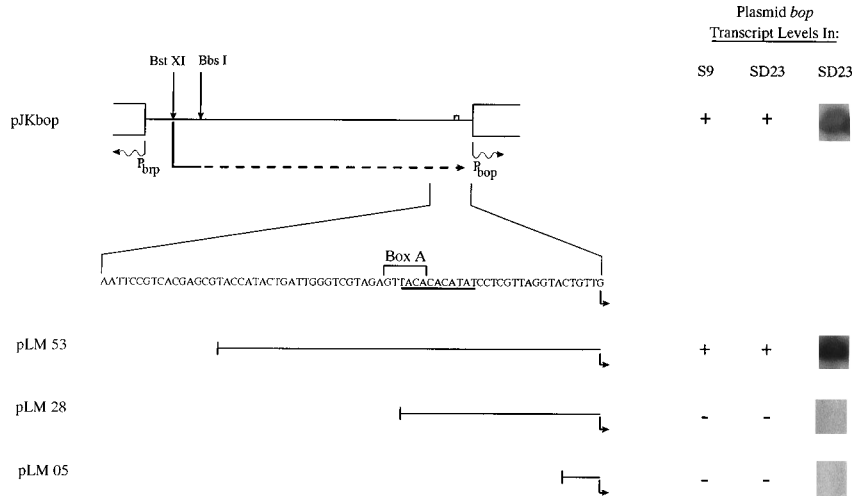

FIG. 5. Deletion analysis of the *bop* promoter region. The 810-bp *Ava*II fragment containing the *bop-brp* intergenic region and N-terminal coding regions (open boxes) is diagrammed above, with the rightward nested deletions from *Bst*XI indicated by the broken arrow. Wavy arrows indicate transcription start points. The DNA sequence of the *bop* promoter region is given below the diagram, with the putative promoter element labeled box A, the alternating purine-pyrimidine sequence underlined, and the transcription start point indicated by an arrow. The promoter region sequences present in three members of the pLM deletion series are indicated by horizontal lines. The transcriptional activities of the *bop* promoter (P_{bop}) on pJKbop and pLM53, -28, and -05 in S9 and SD23 (with corresponding autoradiograms of the cDNA products) are indicated + (active) or − (inactive) on the right. P_{brp}, *brp* promoter.

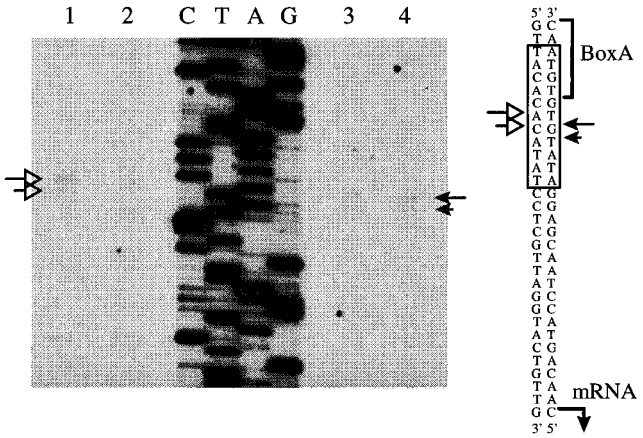

FIG. 6. Structural analysis of the *bop* promoter region. The products of primer extension of *bop* promoter plasmid pCY33 treated with OsO_4 (lanes 1 and 2) and S1 nuclease (lanes 3 and 4) and sequencing reaction products (lanes G, A, T, and C) with the same primer. Lanes 1 and 4 contained the primer extension analysis products with supercoiled pCY33 isolated from late-growth-stage S9, while lanes 2 and 3 contained products with partially-relaxed-form pCY33. The DNA sequence of the *bop* promoter region in double-stranded form is given on the right, with the promoter boxA element bracketed, the 11-bp alternating purine-pyrimidine sequence boxed, and the transcription start point marked by an arrow labeled mRNA. The locations of the primer extension stop sites in lanes 1 and 4 (and the nucleotide sequence) are indicated by open and closed arrows, respectively.

cultures than with stationary-phase cultures (Fig. 3A, lanes 6 and 7). These results are consistent with the requirement of highly negative DNA supercoiling for *bop* transcription.

The *bop* promoter activity on a plasmid in NRC-1, S9, and S9 *bop*, *brp*, and *bat* mutants. To study the contextual and genetic requirements for *bop* gene transcription in more detail, we cloned a 601-bp fragment containing the divergent *bop* and *brp* promoters, amplified by PCR, into the 1.8-kb *Halobacterium* strain GRB plasmid pGRB1 (16); the resulting plasmid, pCY33 (42), was introduced into NRC-1, S9, S9 *bop*, S9 *brp*, and S9 *bat* strains by transformation. Southern hybridization analysis showed that pCY33 did not integrate into the *H. halobium* genome (data not shown). Transcription from the *bop* promoter on the plasmid was measured by primer extension with an oligonucleotide complementary to vector sequences downstream of the *bop* promoter. By comparing the size of the *bop* cDNA produced with a sequencing ladder generated by the same primer on the pCY33 template, the transcription initiation site on the plasmid was shown to be identical to the previously characterized start site on the chromosome (Fig. 4A). The message level detected from the plasmid promoter for S9 was higher than that for NRC-1 (Fig. 4, lanes 1 through 4), as was also observed for the chromosomal *bop* promoter (Fig. 2). In separate reactions, an oligonucleotide complementary to the *bop* transcript from both the plasmid and chromosome was used for primer extension analysis (data not shown). The results indicated approximately equal levels of transcription from both loci, which was expected because of the low (two to three) copy number for pCY33, as determined by Southern hybridization (data not shown).

We also examined the effects of *bop*, *brp*, and *bat* mutations on the activity of the *bop* promoter on pCY33 by primer extension analysis. Pum$^-$ mutants SD23 (S9 *bop*::IS*H1*), SD27 (S9 *brp*::IS*H2*), and SD20 (S9 *bat*::IS*H1*) (42) were transformed with pCY33, and transcript levels were assayed by primer extension analysis (Fig. 4A). Message was observed at high levels for the *bop* mutant (SD23; Fig. 4A, lanes 5 and 6) but was barely detectable for the *brp* mutant (SD27; lanes 7 and 8) and was undetectable for the *bat* mutant (SD20; lanes 9 and 10). These results are consistent with the requirement of an intact *brp-bat* gene region on the chromosome for transcription from the *bop* promoter on a plasmid. Therefore, it shows that the putative regulatory genes, *brp* and/or *bat*, are able to activate transcription when the *bop* promoter is located in *trans.*

The effect of novobiocin on *bop* transcription from pCY33 in SD23 was determined by primer extension analysis of RNA isolated from cultures grown in the presence of 0.005 to 0.1 μg of novobiocin per ml. At these low concentrations, novobiocin has no effect on plasmid copy number (data not shown). The results of primer extension show that *bop* transcription from this plasmid is inhibited by novobiocin to essentially the same degree as the chromosomal gene in S9 is (compare Fig. 4B with Fig. 2D).

Deletion analysis of the *bop* promoter. To determine the extent of the region upstream of the *bop* gene necessary for *bop* promoter function, we carried out deletion analysis, starting from a *Bst*XI site located ~480 bp upstream of the *bop* transcription start point in pJKbop, which is similar to pCY33 but has replication ability in both *H. halobium* and *E. coli* (Fig. 5). Seven plasmids with deletions of the *bop* promoter region, pLM435, pLM365, pLM245, pLM145, pLM53, pLM28, and pLM05 (the numbers indicate the number of nucleotides remaining upstream of the transcription start point), were introduced into *H. halobium* S9 and SD23 (S9 *bop*::IS*H1*) by transformation. RNAs were prepared from these strains and analyzed by primer extension analysis to quantify the *bop* message initiated at plasmid promoters. The results show that transcription occurred in strains carrying pLM53 but not pLM28 (Fig. 5), indicating that a region of at least 53 bp upstream of the transcription start point, but not 28 bp, is sufficient for *bop* promoter activity.

Unusual DNA structure in the *bop* promoter. The *bop* promoter region contains an 11-nucleotide-long alternating purine-pyrimidine sequence, TACACACATAT, centered 23 nucleotides 5′ to the transcription start site, which had been suggested to adopt a Z-DNA structure (6). To test this hypothesis, we treated pCY33 DNA isolated from S9 with S1 nuclease and OsO_4, which are specific for single-stranded regions usually observed at B-Z junctions (21), and used an oligonucleotide hybridizing nearby for primer extension analysis. The same oligonucleotide was also used to generate sequencing ladders. The results in Fig. 6 show that the alternating purine-pyrimidine sequence is susceptible to both OsO_4 (lane 1) and S1 nuclease (lane 4) within the central region. No reaction was observed with partially relaxed pCY33 (Fig. 6, lanes 2 and 3) or linearized plasmid DNA (data not shown), indicating that DNA supercoiling is required. These results indicate that the alternating purine-pyrimidine sequence in the *bop* promoter is capable of adopting a non-B-DNA structure similar to that of Z-DNA under superhelical stress.

DISCUSSION

Several previous studies have focused on the transcription of the *bop* gene of *H. halobium* (11). We previously showed that transcription starts only 2 nucleotides upstream of the structural gene and is induced under microaerobic conditions (6, 43). We also found that transcription is greatly reduced by novobiocin at concentrations subinhibitory for growth, suggesting a role for DNA supercoiling in *bop* transcription (43). Betlach and coworkers showed the involvement of two genes upstream of *bop*, *brp* and *bat*, in *bop* gene expression (1, 14, 15,

24, 25, 36). In this report, we have confirmed that *bop* transcription is sensitive to DNA supercoiling by novobiocin titration experiments. We have shown that when the *bop* promoter is cloned on a plasmid, its activity is similar to that at the normal chromosomal location in several strains and in the presence of novobiocin. Deletion analysis has been used to show the requirement of more than 28 bp but less than 54 bp upstream of the *bop* transcription start site for promoter activity. An 11-bp alternating purine-pyrimidine sequence in the functional promoter region has been shown to be in a supercoiling-dependent Z-DNA-like structure. This result suggests the involvement of unusual DNA structure in supercoiling-stimulated *bop* transcription.

The results of experiments using novobiocin represent strong evidence for the stimulation of *bop* transcription by DNA supercoiling. For example, the transcriptional activities of the *bop* promoter region on both the chromosome and plasmid were sensitive to concentrations of novobiocin subinhibitory for growth, and these low concentrations of novobiocin were shown to decrease plasmid linking deficiency, most likely leading to the general relaxation of DNA supercoiling (Fig. 2 to 4) (42). For *bop* transcription from constructed plasmids, the results further demonstrate that the effects of novobiocin are mediated at the level of *bop* promoter activity since only 45 bp at the 5′ end of the *bop* gene are present on pCY33.

The observed effect of novobiocin on DNA supercoiling most likely results from the inhibition of a eubacterium-type DNA gyrase in *H. halobium*. Previously, DNA gyrase genes have been cloned from a novobiocin-resistant mutant of the related halophile, *Haloferax volcanii* (20), and the relaxation of another *Halobacterium* plasmid, pGRB1, by novobiocin treatment has also been reported before (27, 37). The observed effects of novobiocin on transcription were not mediated via inhibition of DNA replication, since the copy numbers of the chromosomal *bop* gene and plasmid pCY33 remained unchanged, as judged by Southern hybridization (data not shown). However, novobiocin did decrease the growth rate slightly; therefore, we cannot rule out this additional indirect effect on transcription (4).

A complicating factor encountered during the analysis of the effect of novobiocin on gene expression in *E. coli* was a transient increase in DNA supercoiling at low novobiocin concentrations due to the induction of DNA gyrase synthesis (12). A similar phenomenon probably also occurred in *H. halobium*, as suggested by the slightly increased transcription at the lowest novobiocin concentration (Fig. 3).

Transcriptional analysis of *bop* in Pum$^-$ mutants, including *bop*, *brp*, and *bat* mutants, suggested that the *bat* gene product is involved in the activation of *bop* and *brp* gene expression both in S9 (Fig. 3 and 4) (42) and in a wild-type strain similar to NRC-1 (24). Insertions into the *bat* gene (e.g., in SD20) completely abolished the transcription of both *brp* and *bop*. A possible function for the putative Bat protein was suggested by the sequence similarity to the *nifL* gene product from *Klebsiella pneumoniae* and *Azotobacter vinelandii* (about 30% identity in a 150-amino-acid region) (23, 43). This finding suggested that the *bat* gene product plays a role similar to that of the *nifL* gene product, which may respond to oxygen availability by either a direct or an indirect mechanism (9, 14, 23). In this context, it is interesting that the *nifL* promoter is sensitive to DNA supercoiling in vitro (7).

Mutants with insertions in the *brp* gene region (e.g., SD27) produced barely detectable levels of *bop* mRNA (Fig. 4). However, the role of *brp* in *bop* gene expression is not yet clear. The transcription of *brp* has been shown to be induced by high-intensity light in a wild-type strain, suggesting that the predicted Brp protein functions as a receptor or a transmitter of the light signal (36). It is possible that the Brp protein, which is very hydrophobic, interacts with retinal in the membrane and modulates bacterio-opsin synthesis (38). It has been observed that *brp* (and *bat*) mutants display normal phototactic behavior, ruling out an absolute requirement for *brp* in retinal synthesis (38a). A possibility yet to be ruled out is that *brp* mutants are Pum$^-$ because of a polar effect on *bat*, but not because *brp* is directly involved in *bop* gene regulation.

The observed activity of the *bop* promoter on pCY33 suggested that the *brp* and/or *bat* regulatory gene can activate transcription in *trans*. This is consistent with the results of recent experiments in which the introduction of the *bat* or *brp* gene on a multicopy plasmid was shown to complement chromosomal mutations (14). Deletion analysis of the *bop* promoter region (Fig. 5) suggested that the site for activation of transcription by the products of the *brp-bat* gene region (directly or indirectly) resides near the *bop* transcription start point. This site may be within the region 28 to 53 bp upstream of the transcription start site, which contains a sequence related to sequences near other oxygen-regulated genes, e.g., *blp* and *brp* (15). However, the possibility of additional sites further upstream of the transcription start point was suggested by the reduced activity of the *bop* promoter in pLM53 compared with those of plasmids containing larger upstream regions, e.g., pJKbop, as well as the isolation of a partially Pum$^-$ mutant with an insertion of IS*H2* 100 bp upstream of the transcription start point (6).

The mutation in S9 resulting in a high constitutive level of transcription remains enigmatic. No differences in sequence could be detected in the *bop-brp* intergenic region between S9 and NRC-1 (42). One observation made during our studies (data not shown) (42) was that the *brp* mRNA level is higher in S9, suggesting that the *bat* mRNA level is also higher in this strain. This could result in higher levels of Bat and/or Brp protein and constitutive activation of *bop* transcription. However, concentrations of novobiocin reducing *bop* mRNA levels substantially (by 90% or more) showed a smaller (50%) drop in *brp* transcription in S9, suggesting that there is no direct correlation between *bop* and *brp* expression, at least at the transcriptional level. A second possibility is that the S9 *bat* (or *brp*) gene contains a mutation resulting in increased activation of transcription, similar to the *crp** allele of the catabolite activator gene in *E. coli* (13). Gropp and Betlach (14) have recently presented some genetic evidence in support of this hypothesis. A third possibility supported by our observations is that a mutation in the regulation of DNA supercoiling in S9 (42) is responsible for increased transcription of *bop*. The finding of reduced effectiveness of 0.1 mg of novobiocin per ml in inhibiting *bop* transcription in S9 (50%) compared with that in NRC-1 (90%) (Fig. 2) is consistent with this suggestion. A final possibility is that S9 lacks an unidentified repressor of the *bop* gene.

One of the most interesting findings in our studies was that DNA supercoiling promotes the formation of an unusual, non-B-DNA structure just upstream of the *bop* transcription start point (Fig. 6). This structure was located within an 11-bp alternating purine-pyrimidine sequence overlapping the putative TATA-like box A element of archaeal promoters (17, 31). Both S1 nuclease and OsO_4, which are specific for single-stranded regions at B-Z junctions, reacted to the central region of the alternating purine-pyrimidine sequence in supercoiled DNA. A likely possibility is that a part of the 11-bp region is in a dynamic equilibrium between B and Z conformations, as was observed at another region containing $d(CA/TG)_n$ repeats

(19). The likelihood for a B-Z junction at any particular point in time is greatest near the center of the repeat sequence, resulting in susceptibility to S1 nuclease and OsO_4 at the center. Alternatively, our results are consistent with the formation of a bend or kink with appreciable single-stranded character without Z-DNA formation. However, the possibility of a supercoiling-promoted cruciform structure or a partially denatured region in the *bop* promoter is unlikely due to the lack of a palindromic sequence and the occurrence of unreactive regions with similar AT contents nearby. Understanding the detailed nature of this unusual DNA structure and evaluating its possible involvement in promoter function, protein binding, and regulation of transcription will require further analysis.

In conclusion, we have provided a considerable amount of evidence showing that *bop* transcription requires DNA supercoiling. Together with evidence from eubacteria (8, 18) and eukaryotes (10), the sensitivity of transcription to DNA supercoiling has been demonstrated in all major phylogenetic groups. In eubacteria, detailed mechanistic studies have shown that DNA supercoiling can modulate transcription via DNA bending and looping (30, 41), by DNA twisting (2, 40), or by acting directly on RNA polymerase-promoter interactions (3, 26). In *H. halobium*, an additional possibility is that DNA superhelical tension and high salt concentrations contribute to the induction and stabilization of unusual DNA structures, such as left-handed Z-DNA. It remains to be proven if any unusual DNA structures are necessary for transcription or have been recruited for novel mechanisms of gene regulation.

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