JOURNAL OF BACTERIOLOGY, Aug. 1991, p. 4692–4699
0021-9193/91/154692-08$02.00/0

Vol. 173, No. 15

Expression of the *bop* Gene Cluster of *Halobacterium halobium* Is Induced by Low Oxygen Tension and by Light

RICHARD F. SHAND† AND MARY C. BETLACH*

Department of Biochemistry and Biophysics, University of California, San Francisco, California 94143

Received 4 February 1991/Accepted 23 May 1991

The *bop* gene cluster consists of at least three genes: *bop* (bacterio-opsin), *brp* (bacterio-opsin-related protein), and *bat* (bacterio-opsin activator). We have quantitated transcript levels from these genes in a wild-type and bacterioruberin-deficient mutant of *Halobacterium halobium* under conditions which affect purple membrane synthesis. In wild-type cultures grown under high oxygen tension in the dark, *bop* and *bat* transcript levels were low during steady-state growth and then increased ~29- and ~45-fold, respectively, upon entry into stationary phase. *brp* gene transcription remained very low and essentially unchanged under these conditions. In addition, exposure of wild-type cultures growing under high oxygen tension to 30,000 lx of light stimulated expression of all three genes, especially *brp*. In contrast to the wild-type, transcription from all three genes in the bacterioruberin mutant was very high during steady-state growth under high oxygen tension in the dark. Cultures of the bacterioruberin mutant were shifted at early stationary phase to low oxygen tension to determine whether oxygen concentrations lower than those present in stationary phase would induce transcription of the *bop* gene cluster in this strain. Indeed, transcription was induced, suggesting that the *bop* gene cluster is not completely uncoupled from regulation by oxygen tension in the bacterioruberin mutant. From these data, we propose a regulatory model involving two different mechanisms: (i) *bat* gene expression is induced under conditions of low oxygen tension and the *bat* gene product activates *bop* gene expression and (ii) light induces *brp* transcription, which stimulates or modulates *bat* transcription.

Bacterio-opsin is the sole protein found in the purple membrane of the extremely halophilic, photosynthetic archaebacterium *Halobacterium halobium*. The complex of this protein with the chromophore retinal constitutes bacteriorhodopsin (BR), which mediates photophosphorylation (5, 11) by generating a transmembrane electrochemical potential via light-driven proton pumping (22). The structure and function of BR have been extensively studied (4, 12), but less is known about the genetics and physiology of bacterio-opsin (*bop*) gene expression in vivo.

Characterization of some 50 spontaneous Bop mutants (1, 2, 6, 13) has shown that the gene encoding bacterio-opsin is flanked by at least two other genes that are involved in its expression: the bacterio-opsin-related protein gene (*brp*) and the bacterio-opsin activator gene (*bat*) (1, 14; Fig. 1A). All three genes have been sequenced, and their transcriptional start sites have been determined (1, 8, 9). The *brp* gene is located 526 bp upstream of the *bop* gene and is transcribed in the opposite orientation (1). The *bat* gene is located 1,602 bp upstream of the *bop* gene and is transcribed in the same orientation as the *brp* gene (14). There is no evidence supporting the existence of a polycistronic message arising from the *brp* and *bat* genes.

Mutants containing insertion mutations in either the *brp* gene, the *bat* gene, or the intergenic region between the *bop* and *brp* genes have greatly reduced *bop* gene expression, varying from 0 to 23% of the parental levels (13). While some of the effects on *bop* gene expression caused by these insertion mutations have obvious explanations (e.g., disruption of the putative *bop* gene promoter), disruption of *brp* gene expression reduces *bop* gene expression through an unknown mechanism. In addition, these studies suggest that the *bat* gene product may be involved in activating *brp* and *bop* gene expression (13). Secondary structure predictions indicate that the putative *bat* protein would consist of alternating alpha helices and beta sheets characteristic of soluble proteins (14), whereas the putative *brp* protein would consist of six to seven hydrophobic alpha helices of sufficient length to span the membrane (3). It has been suggested that the *brp* protein may act as a membrane-bound sensor or be involved in the assembly of purple membrane (3). Neither of the putative *brp* or *bat* proteins have yet been detected in halobacterial cells, since there is no available assay. Transcript levels from the *bop* gene cluster in wild-type and Bop mutants have been measured and compared only at a single point during the growth cycle (approximately late exponential/early stationary phase) in aerobically grown cultures (13). *brp* and *bat* transcript levels from wild-type cultures grown aerobically in complex medium under ambient light were 2 and 4%, respectively, of *bop* levels (13).

Oxygen tension, light intensity, and retinal synthesis all have been implicated as affecting purple membrane synthesis (20, 22, 26, 27). In addition, there may be some level of interaction between the synthesis of purple membrane and the major C_{50}-isoprenoid, bacterioruberin. Bacterioruberins are a group of 4 to 6 C_{50}-hydroxylated isoprenoids that are responsible for imparting the red pigmentation seen in *H. halobium*. Synthesis of bacterioruberin may impinge upon purple membrane synthesis since (i) bacterioruberin shares a common biosynthetic pathway with retinal (28) and (ii) some Bop Rub double mutants spontaneously partially revert to Rub^+ if the Bop mutation is located within or close to the *bop* gene (23).

We have begun a systematic quantitation of *bop* gene cluster transcript levels in a wild-type strain and in a bacterioruberin-deficient mutant grown under conditions of either high or low oxygen tension and either in the light or in

* Corresponding author.
† Present address: Department of Biological Sciences, Northern Arizona University, Flagstaff, AZ 86011.

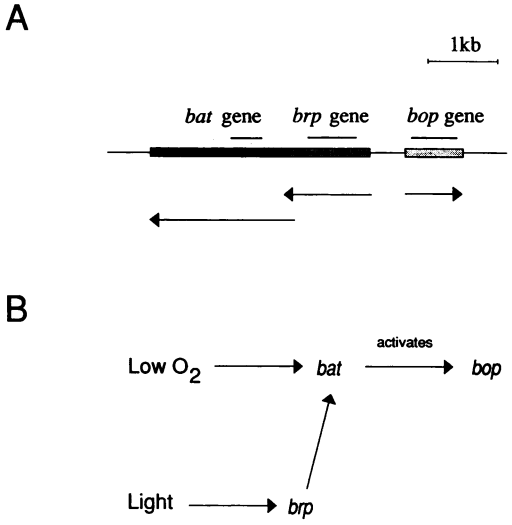

FIG. 1. (A) Map of the *bop* gene cluster. Positions of the genes are indicated by the thick bars. Internal DNA fragments of the three genes which were used to generate RNA probes used for Northern blot analyses are indicated as solid lines above the map. The extent and direction of transcription from the three genes are indicated by the arrows located beneath the map. (B) Proposed model for regulation of the *bop* gene cluster. Low oxygen tension induces *bat* gene expression, and the *bat* protein (which may not be the final effector) activates *bop* gene expression. High light intensity induces *brp* transcription, which stimulates and/or modulates *bat* transcription. The placement of an arrow from *brp* to *bat* is based on previous transcriptional studies of mutants containing insertions in the *brp* gene (3, 13).

the dark. We sought to determine whether *bop*, *brp*, and *bat* gene expression are constitutive or inducible, to determine whether the *brp* and *bat* genes are coordinately regulated with the *bop* gene, and to obtain insights as to the mechanism(s) whereby the putative *brp* and *bat* gene products interact with the *bop* gene cluster.

MATERIALS AND METHODS

Bacterial strains, medium, and growth conditions. *H. halobium* strains used in this work were the wild-type strain NRC817 and its spontaneous bacterioruberin-deficient derivative II-7 (23). The location and nature of the bacterioruberin mutation are unknown, but phenotypically, colonies of II-7 appear light purple on solid medium due solely to purple membrane. II-7 is the parent of many of the spontaneous *bop* mutants that were critical in identifying putative *bop* regulatory genes (23).

Halobacterial complex medium contained, per liter, 245 g of NaCl, 20 g of $MgSO_4 \cdot 7H_2O$, 3 g of sodium citrate $\cdot 2H_2O$, 2 g of KCl, 15 g of peptone (Oxoid), and 43.75 mM Tris hydrochloride (pH 7.2). Solid medium contained 1.5% agar (Oxoid). Viable counts were performed by the spread plate technique. Optical density at 600 nm (OD_{600}) was measured using a Beckman DU50 spectrophotometer. Cultures were grown routinely at 37°C in a shaking water bath under ambient light and subcultured twice from early exponential phase before subculturing to an initial OD_{600} of ~0.01 at the start of each experiment. For the high-oxygen-tension experiment (see Fig. 3 to 5), high oxygen tension is defined as incubation at 37°C in cotton-plugged Erlenmeyer flasks at approximately 350 rpm with a culture volume to flask volume of 1:4. Cultures were incubated in either a shaking water bath with the flasks covered with aluminum foil or in the phototrophic chamber (see Fig. 2) with the flasks exposed to 30,000 lx of light.

For shifts to low oxygen tension (see Fig. 6 to 8), a culture was grown under high oxygen tension and ambient light to early stationary phase. Fifty milliliters of culture was transferred to each of two Monogro culture flasks (Wheaton) (warmed to 37°C; one covered with aluminum foil). To generate low-oxygen conditions, flasks were closed with natural rubber sleeve stoppers (Wheaton no. 224097), sealed with three wraps of beeswax-coated string, and tied securely. Two 16-gauge needles were passed through each sleeve stopper to allow for a small amount of air exchange. The sleeve stoppers were loosely covered with aluminum foil to prevent photooxidation of the rubber. The flasks were placed horizontally in the phototrophic chamber and shaken at 100 rpm. Samples (~2 to 3 ml) were removed through the septa with syringes equipped with 25-gauge needles.

Phototrophic chamber. Figure 2 is a schematic of the phototrophic chamber that delivers 30,000 lx of light to the cultures. Incubating cultures horizontally in Monogro culture flasks created a relatively large surface area (~100 cm^2) and reduced culture depth to only a few millimeters, allowing homogeneous exposure and maximum penetration of light. Cultures could also be grown with rapid shaking (high oxygen tension) and exposed to 30,000 lx of light in standard Erlenmeyer flasks. To minimize infrared heating of both the chamber and the contents of the culture flasks, a wide-band hot mirror was used which reflected ~90% of the infrared energy (750 to 1,200 nm), absorbed all of the UV energy (below 400 nm), and transmitted ~85% of the visible light (450 to 675 nm) generated by the lamps. The light intensity requirements are described by Oesterhelt and Krippahl (20); illumination was measured with a Minolta AutoMeter III light meter equipped with a flat diffuser.

Small-scale RNA extraction and Northern (RNA) blot analysis. Total RNA was extracted from 1.8-ml samples by using a modification of the RNAzol procedure (Cinna Biotech). Culture samples were harvested in microcentrifuge tubes at 13,000 rpm for 10 min in a model MC-150 Tomy microfuge at room temperature. The supernatant was decanted, and any medium remaining above the pellet was removed by aspiration. Cell pellets were quickly frozen on dry ice and stored at −65°C until extracted. RNAzol reagent (1.6 ml) was added directly to frozen (−70°C) cell pellets and vortexed vigorously for 15 s. The remainder of the procedure is as described in the product brochure. Air-dried RNA samples were resuspended in 25 μl of diethyl pyrocarbonate-treated water (15) and stored at −65°C. Samples of 30 to 50 μg of total RNA were routinely extracted from 1.8 ml of stationary-phase culture.

RNA (2 μg) from each time point was heated to 65°C in 50% formamide–2.2 M formaldehyde–50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)–1 mM EDTA. RNA samples were electrophoresed on 1.2% vertical agarose gels (0.8 mm thick) at 75 V as described previously (1). RNA was blotted onto Hybond-N nylon membrane (Amersham) and cross-linked by UV light (Stratalinker; Stratagene). ^{32}P-labeled RNA probes were synthesized in vitro, using an SP6 polymerase riboprobe kit (Promega Biotec) from DNA templates containing specific internal fragments of the *bop*, *brp*, and *bat* genes (13; Fig. 1A). Prehybridization, hybridization, and wash conditions were as described by Melton et al. (17) except that prehybridization was performed overnight and the optimum prehybrid-

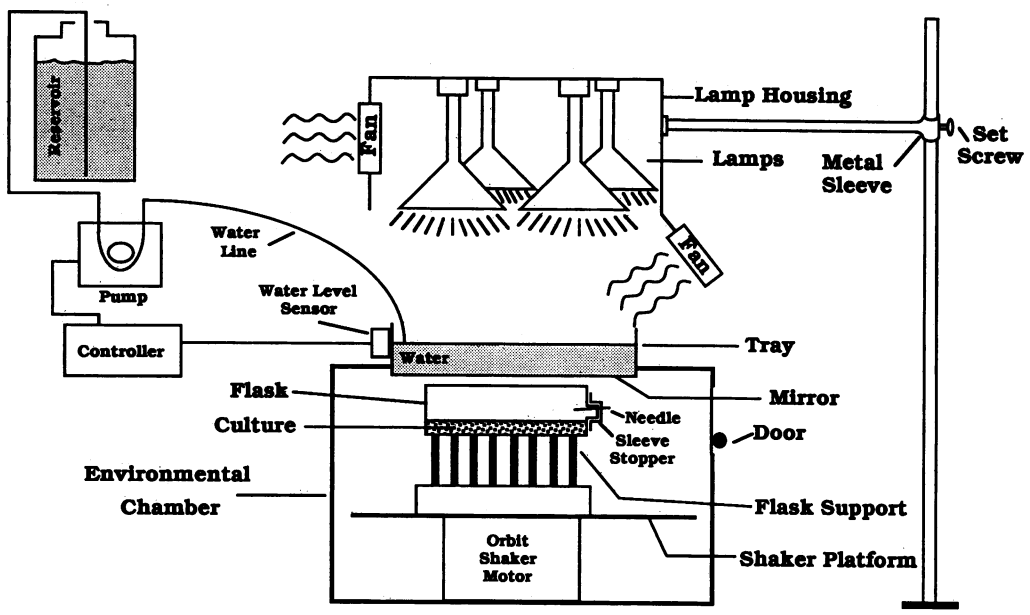

FIG. 2. Phototrophic chamber. The light source was a two-by-two array of 150-W tungsten spot lamps (GE 150R/SP) mounted directly above the chamber. Cultures were illuminated at light intensities described in Oesterhelt and Krippahl (20). These levels were 50 to 100 mW/cm^2, which are equal to 47 to 93 W/ft^2. Xenon/mercury lamps of the type described previously (20) generate approximately 30 lm/W. This equals 1,410 to 2,790 foot-candles (lumens per square foot) or 15,171 to 30,020 lx (lumens per square meter, the equivalent SI unit). Excess heat was removed by evaporating water from a watertight Plexiglas tray (25 by 25 by 3 cm) that was mounted in the plastic lid of a Lab-Line model 3528 environmental shaker. The bottom of the tray was composed of an optically coated, wide-band hot mirror (Optical Coating Laboratory, Inc.) installed with the coated surface down. The water level in the tray was kept constant by using an infrared RM-1 reservoir monitor (Instruments for Research and Industry) attached to the side of the tray and connected to a reservoir through a peristaltic pump.

ization, hybridization, and wash temperatures were determined to be 80°C. Kodak X-Omat-R AR5 film was used for autoradiography of the filters. Exposure times were optimized so that blots containing high levels of message did not saturate the film. mRNA levels were quantitated by scanning the autoradiograms with a Joyce Loebl Ephortec densitometer (626-nm filter) and integrating the areas under the peaks (see legend to Fig. 4).

Quantitation of BR. Samples (400 μl) from each time point were harvested as described above, quickly frozen, and stored at −65°C. Pellets were resuspended and lysed in 100 μl of DNase A (40 μg/ml; Sigma) in H_2O. The lysate was mixed well until homogeneous and no longer viscous. For II-7 cultures, BR content was quantitated spectrophotometrically by scanning the lysed samples from 400 to 700 nm on a Beckman DU50 spectrophotometer. Bacteriorhodopsin concentrations were determined according to the molar extinction coefficient for purple membrane (ε_{568} = 63,000 M^{-1} cm^{-1}; 24). For NRC817 cultures, the presence of bacterioruberins interferes with the spectrophotometric quantitation of BR (28). A freshly prepared 2 M stock solution of NH_2OH was made by mixing equal volumes of 4 M NH_2OH and 4 M NaOH. One-tenth volume of the 2 M NH_2OH solution was added in the dark to the lysed NRC817 sample, and an absorption spectrum was taken on a Shimadzu UV160U spectrophotometer. The sample was bleached (i.e., the chromophore was removed from BR) at 3-min intervals, using 6.5 × 10^5 lx of light produced by a 150-W illuminator (Cole-Parmer) equipped with a yellow filter and a heat filter. Spectra were taken until there was no further decrease in absorption and the amount of BR determined from the difference between the bleached and unbleached spectra at 568 nm. Total protein was determined by using the Bio-Rad protein assay reagent with bovine serum albumin as the standard.

RESULTS

Growth curves from high-oxygen-tension cultures. *H. halobium* strains NRC817 and II-7 were grown under conditions of high oxygen tension in the dark and in 30,000 lx of light. Growth was monitored both by optical density (Fig. 3A) and by viable counts (Fig. 3B). There was very little difference in generation times during exponential growth (see legend to Fig. 3), showing that NRC817 and II-7 have nearly identical growth rates and that 30,000 lx of light had no effect on growth rate under conditions of high oxygen tension in a complex medium. In addition, all cultures reached approximately the same cell density at early stationary phase (1 × 10^9 to 2 × 10^9 CFU/ml). In stationary phase, however, II-7 reached two- to threefold-higher OD_{600} levels than did NRC817 (Fig. 3A). Viable counts and optical densities correlated well in the NRC817 cultures, as both stopped increasing at approximately the same time. In contrast, optical densities in the II-7 cultures continued to increase well after viable counts had stopped increasing (compare Fig. 3A and B).

Transcript levels from high-oxygen-tension cultures. Figure 4 shows *bop*, *brp*, and *bat* transcript levels from the cultures grown under high oxygen tension shown in Fig. 3. Relative comparisons among transcript levels from the same gene can be made, since RNA samples were blotted, prehybridized, and hybridized in parallel with the same ^{32}P-labeled riboprobe. During growth of NRC817 under conditions of high oxygen tension in the dark, *bop* and *bat* transcript levels followed the same pattern of expression (Fig. 4A and C):

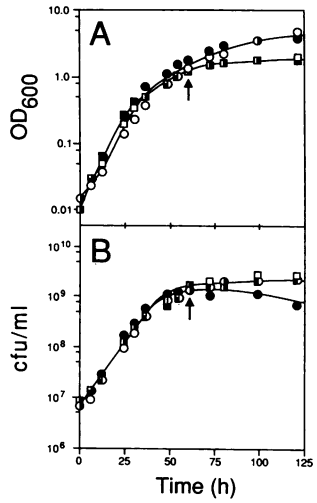

FIG. 3. Growth curves of NRC817 and II-7 grown under high oxygen tension in the light and in the dark as determined by OD_{600} (A) and by viable counts (B). For clarity, two curves are drawn in panel A, one representing the NRC817 cultures, the other representing the II-7 cultures. Similarly, since generation times were essentially identical, only one curve is drawn in panel B until stationary phase, when a second curve appears showing the deviation of the II-7 dark culture from the other three. Viable counts were performed in triplicate and averaged. Symbols: ■, NRC817 in the dark; □, NRC817 in the light; ●, II-7 in the dark; ○, II-7 in the light. Generation times determined from the growth curves in panel B are 5.6, 5.5, 5.25, and 5.7 h, respectively. The arrows indicate early stationary phase and appear as reference markers in Fig. 4 and 5.

transcript levels were very low throughout mid- and late exponential phase, began to rise during early stationary phase (~72 h), reached their highest levels at 100 h, and then declined. The fold differences in *bop* and *bat* transcript levels between 60 h (indicated by arrows in Fig. 4) and their maxima were ~29 and ~45-fold, respectively. *brp* transcripts were not detectable until 80 h and then, unlike *bop* and *bat* levels, increased only slightly (Fig. 4B).

Exposure of cultures of NRC817 growing under conditions of high oxygen tension to 30,000 lx of light resulted in a steady increase in *bop*, *brp*, and *bat* transcript levels during exponential phase and early stationary phase (Fig. 4A through C). This increase in transcription contrasts with the very low transcript levels observed during these stages of growth in the dark culture. Even though *bop* transcript levels were more abundant during exponential phase in the light culture than in the dark culture, *bop* mRNA levels from the light culture reached approximately the same maximum in the same amount of time as the *bop* transcript levels in the dark culture (Fig. 4A). *bat* transcripts from the culture grown in the light were also abundant during stationary phase, but reached approximately the same maximum some 40 h earlier than *bat* transcripts from the dark culture (Fig. 4C). The greatest effect of 30,000 lx of light during growth under high-oxygen-tension conditions was on *brp* transcript levels. While only just detectable in stationary phase of the dark culture, *brp* mRNA levels rose dramatically during exponential phase and early stationary phase in the light and remained markedly elevated (Fig. 4B). The difference between maximal *brp* transcript levels from the dark culture and the light culture was eightfold.

The bacterioruberin-deficient mutant II-7 grown under conditions of high oxygen tension in the dark differed in its *bop* gene cluster transcription patterns from its wild-type parent in two ways (Fig. 4D through F). First, transcript levels from all three genes were markedly elevated during exponential growth. *bop* transcript levels were nearly maximal at about 40 h and remained elevated, with only a slight decline from 100 to 125 h (Fig. 4D). *brp* transcript levels were also maximal at 40 h and then declined (Fig. 4E). *bat* transcripts were nearly maximal at about 60 h and then

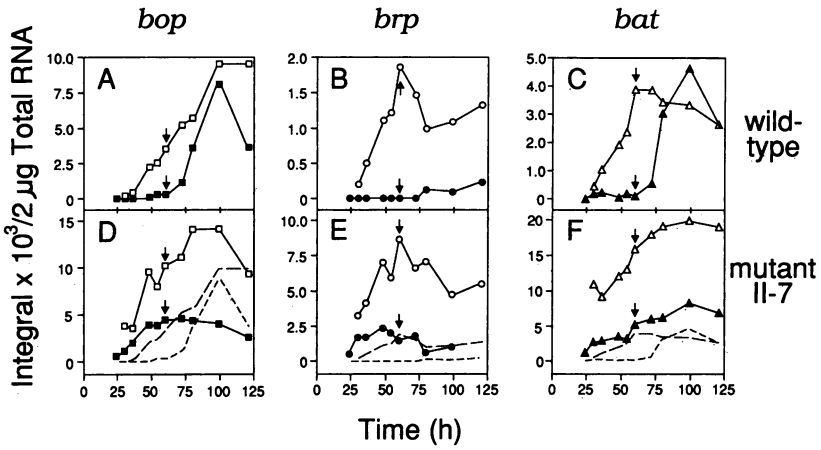

FIG. 4. *bop* gene cluster transcript levels from wild-type (A to C) and mutant II-7 (D to F) cultures grown under high oxygen tension. Open symbols represent mRNA from cultures grown in the light; closed symbols represent cultures grown in the dark. Symbols: □, ■, *bop* mRNA levels (A and D); ○, ●, *brp* mRNA levels (B and E); △, ▲, *bat* mRNA levels (C and F). For comparison of relative levels of transcription between the wild-type and II-7 strains, the fainter long and short dashed lines in panels D through F represent the corresponding *bop*, *brp*, or *bat* transcriptional pattern from the wild-type strain (A through C) grown in the light and dark, respectively, plotted to the same scale as the mutant data. Autoradiography exposure times of Northern blots were 4 h at room temperature for panels A and D and 3 days at −65°C with an intensifying screen for the remaining panels. Levels of specific mRNAs were quantitated by scanning autoradiograms as described in Materials and Methods. Peak areas (integrals) generated from each scan are plotted against time.

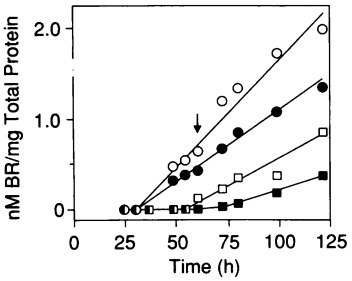

FIG. 5. Quantitation of BR levels from NRC817 and II-7 cultures grown under high oxygen tension in the dark and in the light. Symbols are as described in the legend to Fig. 3.

remained elevated throughout stationary phase (Fig. 4F). Second, the large inductions in *bop* and *bat* transcript levels seen as the wild-type culture approached stationary phase (Fig. 4A and C) were absent in the II-7 culture (Fig. 4D and F). In addition, maximum levels of *brp* and *bat* from the II-7 culture grown in the dark were as high or higher than the maximum *brp* and *bat* levels in the wild-type strain grown in either the dark or the light (Fig. 4E and F).

Exposure of high-oxygen-tension cultures of mutant II-7 to 30,000 lx of light significantly increased transcript levels of all three genes in the *bop* gene cluster (Fig. 4D through F). *bop*, *brp*, and *bat* transcript levels in mutant II-7 rose more rapidly during the exponential and early stationary phases of growth and attained maximum levels that were three- to fourfold higher than the maximum levels attained in the aerobic culture grown in the dark. This is in contrast to what was observed in light-exposed cultures of the wild-type strain, for which maximum levels of *bop* and *bat* were nearly identical to those reached in the dark culture.

BR levels from high-oxygen-tension cultures. Figure 5 compares the amounts of BR from strains NRC817 and II-7 grown under conditions of high oxygen tension in the light and in the dark as shown in Fig. 3. The increase in BR protein levels parallels the increase in *bop* mRNA levels shown in Fig. 4A and D up to 100 h. Both NRC817 and II-7 cultures grown in the light produced detectable BR earlier and at higher levels than did cultures grown in the dark. However, BR levels were two- to threefold higher in strain II-7 than in strain NRC817 under identical growth conditions.

Growth curves of II-7 cultures shifted from high to low oxygen tension. Since strain II-7 lacked the dramatic induction of *bop* transcription seen in wild-type stationary-phase cultures in the dark (compare Fig. 4A and D), we aimed to determine whether oxygen concentrations lower than those present during stationary phase would affect *bop* gene cluster expression. It is well documented that reduced oxygen tension is one condition necessary to cause *H. halobium* to produce large amounts of purple membrane (21, 22). Classical purple membrane-inducing conditions involve growing cultures aerobically under ambient light until early stationary phase, followed by reduced aeration and increased illumination. Consequently, we mimicked these growth conditions (Fig. 6) to determine whether lower oxygen tension could restore the induction phenomenon in strain II-7.

Figure 6 shows growth curves determined by OD_{600} (Fig. 6A) and by viable counts (Fig. 6B) from cultures grown under high oxygen tension (preshift) and then shifted to low-oxygen conditions in the light and in the dark (postshift). Very large increases in optical densities but not in viable

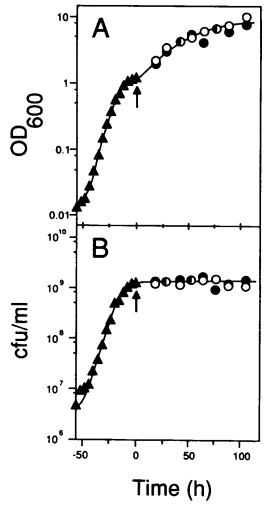

FIG. 6. Growth curves determined by OD_{600} (A) and viable counts (B) of *H. halobium* II-7 grown under high oxygen tension and ambient light and then shifted at early stationary phase (indicated by arrows) to low oxygen tension in the light and in the dark. Symbols: ▲, growth under high oxygen tension (preshift); ○, samples from cultures shifted to low oxygen and high light intensity; ●, samples from cultures shifted to low oxygen and kept in the dark.

counts were observed postshift in both the light and dark cultures (Fig. 6). The large increases in optical density correlated with the production of large amounts of refractory gas vacuoles which were observed under these conditions (25).

Transcript levels of II-7 cultures shifted from high to low oxygen tension. Preshift and postshift levels of *bop*, *brp*, and *bat* transcripts from the II-7 cultures described in Fig. 6 are shown in Fig. 7. Preshift (high oxygen tension) *bop* mRNA levels (Fig. 7A) increased as the II-7 cultures approached and entered stationary phase, as seen previously in high-oxygen-tension dark cultures (Fig. 4D). Upon shift to low oxygen tension, *bop* transcript levels reached their postshift maximum at 18 h in both the dark and light cultures. *bop* transcript levels from the culture shifted to the light reached a slightly higher maximum than did the culture placed in the dark (approximately three- and twofold increases, respectively, above the highest [time 0] preshift level). In the dark culture, postshift *bop* transcript levels remained constant from 18 to 64 h and then declined. In comparison, postshift *bop* transcript levels from the light culture steadily declined from their maximum at 18 h. In addition, the maximum postshift *bop* transcript level from the dark culture shifted to low oxygen tension was approximately twofold higher than the maximum *bop* transcript level from the dark high-oxygen-tension culture (Fig. 7A, dashed curve).

Significantly larger postshift effects were seen on *brp* and *bat* transcript levels than were seen for *bop* transcript levels. Preshift (high oxygen tension) transcript levels from both the *brp* gene and the *bat* gene were relatively low and increased very little from −20 to 0 h (Fig. 7B and C). The largest and most rapid postshift increases in *brp* and *bat* transcript levels occurred in the culture shifted to 30,000 lx of light (~10- and ~37-fold increases, respectively, above the highest [time 0] preshift level). Under these conditions, maximum *brp* and *bat* transcript levels were reached at 18 and 28 h, respectively, remained elevated until 53 h, and then declined, returning to near preshift levels by 105 h.

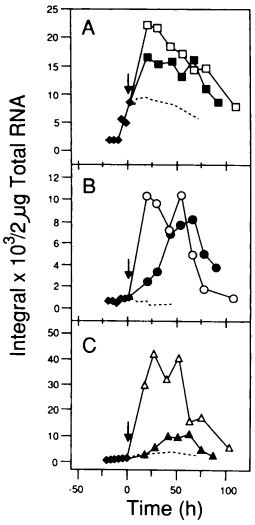

FIG. 7. *bop* gene cluster transcript levels from the cultures shown in Fig. 6. Preshift (high oxygen tension) transcript levels (◆) are averages of two samples. Arrows indicate shift at early stationary phase from aerobic growth to low oxygen tension in the dark (closed symbols) and in the light (open symbols). Symbols: ■, □, *bop* mRNA (A); ●, ○, *brp* mRNA (B); ▲, △, *bat* mRNA (C). The fainter dashed lines are derived from Fig. 4D through F and represent approximate corresponding transcript levels of *bop*, *brp*, and *bat* from II-7 cultures grown in the dark under high oxygen tension. Peak areas (integrals) generated from each scan are plotted against time.

brp and *bat* gene transcript levels also increased in the culture shifted to low oxygen tension and left in the dark (~8- and ~10-fold increases, respectively, above the highest [time 0] preshift level). However, compared with *brp* and *bat* transcript levels from the light culture, the kinetics of the increases were delayed (reaching maximum levels at 64 h), and the magnitudes of the increases were smaller (~8-fold in the dark versus ~10-fold in the light for *brp* and ~10-fold in the dark versus ~37-fold in the light for *bat*). The postshift *brp* and *bat* transcriptional maxima from dark cultures shifted to low oxygen tension were ~8- and ~3-fold higher, respectively, than the highest *brp* and *bat* transcript levels from dark high-oxygen-tension cultures (Fig. 7B and C, dashed curves).

BR levels from II-7 cultures shifted from high to low oxygen tension. Initial postshift BR levels from the II-7 culture shifted to low oxygen and high light intensity were ~4-fold higher than for the shifted culture that remained in the dark (Fig. 8). Similarly, initial *bop* mRNA levels in the culture shifted to the light were elevated above those from the culture kept in the dark (Fig. 7A). At approximately 53 h postshift, BR levels from the dark culture became and remained equal to the BR levels from the light culture.

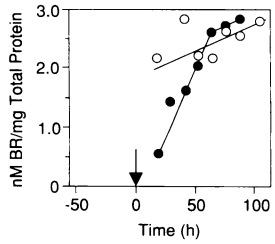

FIG. 8. Quantitation of BR levels from II-7 cultures grown under high oxygen tension to early stationary phase and then shifted (indicated by an arrow) to low oxygen tension in the dark (●) or the light (○). The correlation coefficient (*r*) of the line described by the open circles is 0.50.

DISCUSSION

High oxygen tension. We have quantitated mRNA levels expressed from three genes in the *bop* gene cluster in two strains of *H. halobium* throughout the exponential and stationary phases of growth under high oxygen tension in both the dark and the light. The growth curves of strain II-7 revealed a slight increase in optical density in stationary phase that was not accompanied by an increase in viable counts (Fig. 3). The reason for this difference is unknown, although increased synthesis of purple membrane and gas vacuoles could be contributing factors (25).

bop, *brp*, and *bat* transcript levels in the wild-type strain remained relatively suppressed during steady-state growth in the dark (Fig. 4A through C). As the culture entered stationary phase, *bop* mRNA levels increased 25- to 29-fold above transcript levels observed during exponential growth (Fig. 4A). Yang and DasSarma also have observed an ~20-fold increase in *bop* transcription between mid-exponential-phase and stationary-phase wild-type cultures (29). The *bop* transcriptional profile was paralleled by BR production throughout the majority of the growth cycle (Fig. 4A and 5), which supports the observation that high levels of oxygen repress the synthesis of purple membrane (22). The marked decline in *bop* mRNA in late stationary phase of dark cultures was not paralleled by a decline in BR, suggesting either decreased *bop* mRNA synthesis or increased *bop* mRNA degradation and attesting to the stability of BR. Indeed, Oesterhelt and Stoeckenius report that up to 50% of the total cell membrane surface area can consist of purple membrane in stationary-phase cells (22).

In contrast, *brp* transcript levels in cultures of NRC817 grown in the dark were barely detectable and increased only slightly in stationary phase (Fig. 4B). The *bat* gene transcriptional pattern, on the other hand, was nearly identical to that of the *bop* gene, with *bat* transcript levels increasing 20- to 45-fold above those observed during exponential growth (Fig. 4C). Thus, both *bop* and *bat* transcription are induced during stationary phase in wild-type cultures grown under high oxygen tension in the dark, while *brp* transcription remains relatively unaffected. This induction is most likely due to a decrease in oxygen tension which naturally occurs in stationary phase, although significant contributions by other stationary-phase effects such as depletion of nutrients other than oxygen, a general starvation response, or accumulation of regulatory metabolites cannot be ruled out.

Exposure of cultures of NRC817 growing under high oxygen tension to 30,000 lx of light stimulated transcription from all three genes during steady-state growth, and transcript levels remained elevated during stationary phase. This stimulation was apparent at the earliest steady-state time points (Fig. 4A through C) and indicates that exposure to light mitigates the repressive effect of high oxygen tension seen during steady-state growth.

Previously, we suggested that the *brp* protein may function as a membrane-bound sensor, that the *bat* protein may function as a soluble regulatory molecule (3), and that, as determined from analysis of transcript levels in numerous Bop mutants (13), the *bat* gene product activated *bop* and

brp gene expression. Data in this report support most of these proposed functions and suggest a model (Fig. 1B) in which at least two mechanisms are involved in regulation of *bop* gene expression, one involving oxygen and the other involving light. *brp* transcription is induced by light but not by decreasing oxygen tension, which suggests that the putative *brp* protein may function as a receptor and/or a transmitter of the light signal. Such roles are consistent with the predicted secondary structure of the putative *brp* protein, which consists of six to seven hydrophobic alpha helices of sufficient length to span the halobacterial membrane and two amphipathic alpha-helical cytoplasmic regions (3). On the other hand, *bat* transcription is induced by both light and decreased oxygen tension, and the *bat* gene product may serve as an intermediary in transmission of both signals. As observed by ourselves and others (29), the primary amino acid sequence of the *bat* protein deduced from the nucleotide sequence (14) possesses a region of significant homology with the *nifL* gene product, a putative oxygen sensor found in *Klebsiella pneumoniae* (7). Finally, it is difficult to assess the temporal relationships between *bop*, *brp*, and *bat* gene expression levels during growth under high oxygen tension within our 6- to 12-h sampling time frame. However, in high-oxygen-tension cultures exposed to 30,000 lx of light, *brp* and *bat* mRNA levels reach their maxima at the same time and some 40 h earlier than *bop* mRNA maximum levels (Fig. 4 through C). Moreover, maximal *bop* transcript levels were reached at the same time in both the dark and light high-oxygen-tension cultures (Fig. 4A).

Strain II-7, a bacterioruberin-deficient mutant of the wild-type strain, NRC817, had significantly different patterns of *bop*, *brp*, and *bat* transcription than its parent in cultures grown under high oxygen tension in the dark. The low steady-state levels and large inductions of *bop* and *bat* transcription observed in stationary phase in the wild-type strain were absent in strain II-7 (Fig. 4D and F), indicating that in this mutant, *bop* and *bat* transcription do not respond to decreasing oxygen tension in the same fashion as in the wild-type. In addition, the constantly increasing levels of *bop*, *brp*, and *bat* transcripts observed during steady-state growth under high oxygen tension in the dark suggest that high oxygen tension does not repress *bop*, *brp*, and *bat* transcription in strain II-7. Comparisons of *bop* gene cluster transcriptional patterns between mutant II-7 and the wild type (Fig. 4) reveal that II-7 grown in the dark has higher *brp* and *bat* transcript levels than does the wild-type culture grown in the dark or the light. The maximum *bop* transcript level attained in strain II-7 was about 50% of the maximum attained by the wild type in the dark or the light. These data show that strain II-7 constitutively expresses *bop* gene cluster transcripts during steady-state growth under high oxygen tension in the dark.

Despite the apparent uncoupling of the *bop* gene cluster from regulation by oxygen tension in strain II-7, exposure of cultures of II-7 to 30,000 lx of light during steady-state growth under conditions of high oxygen tension resulted in an increase in transcription from all three genes, similar to that seen in the wild-type strain. However, mutant II-7 attained three- to fourfold-higher maximum levels of *bop* gene cluster transcripts under these conditions than did its wild-type parent.

Strain II-7 differs from its wild-type parent by an uncharacterized mutation which affects bacterioruberin levels. Although the presence of another mutation elsewhere in the genome cannot be fully discounted, our data suggest that the Rub mutation in strain II-7 somehow results in an insensitivity of the *bop* gene cluster to oxygen tension. On the other hand, the *bop* gene cluster in II-7 displays a sensitivity to light similar to that of the wild-type strain, although higher levels of expression were attained. Pfeifer et al. (23) have suggested that there may be some level of interaction between bacterio-opsin and bacterioruberin syntheses, given the fact that Bop mutants with insertions in or near the *bop* gene manifest a concomitant partial phenotypic reversion of bacterioruberin synthesis whereas Bop mutants with inserts further upstream of the *bop* gene do not. Bacterioruberins share a common biosynthetic pathway with retinal, and a decrease in the synthesis of bacterioruberins could conceivably increase retinal synthesis. In turn, an increase in free retinal might signal induction of the *bop* gene cluster. Such a scenario may be the basis for the increased *bop* gene cluster expression observed in strain II-7. Supporting this hypothesis is the observation that addition of exogenous retinal to cultures in which the retinal biosynthetic pathway was blocked by nicotine resulted in a fivefold increase in BR levels (26). In addition, an increase in bacterio-opsin transcription has been seen in a retinal-deficient mutant upon addition of exogenous retinal (10).

Shift to low oxygen tension. We have quantitated transcript levels expressed from the *bop* gene cluster in cultures of strain II-7 grown under high oxygen tension to early stationary phase and then shifted to low oxygen tension and either kept in the dark or exposed to 30,000 lx of light. This was done to examine the apparent uncoupling of the *bop* gene cluster from regulation by oxygen tension in strain II-7 by determining whether oxygen concentrations could be lowered sufficiently to invoke an induction response.

Following the shift to low oxygen and light, which simulated classical conditions for producing large quantities of purple membrane, the levels of all three transcripts (*bop*, *brp*, and *bat*) increased considerably (~3-fold for *bop*, ~10-fold for *brp*, and ~37-fold for *bat*) compared with preshift, high-oxygen-tension transcript levels in the dark. Thus, classical methods used to produce large amounts of purple membrane simultaneously induced transcription of all three genes in the *bop* gene cluster in strain II-7. These results also show that decreased oxygen tension alone can induce transcription from all three genes of the *bop* gene cluster in strain II-7 and to significantly higher levels than those achieved in stationary-phase, high-oxygen-tension cultures.

Despite the significant increases in *bop* gene cluster transcription during shifts to low oxygen tension, addition of 30,000 lx of light resulted in both higher maxima and increased rates of synthesis (although marginal in the case of *bop* expression) for the three genes (Fig. 7). These data confirm that light stimulates expression from the *bop* gene cluster under low as well as high oxygen tension. Furthermore, even though oxygen tension decreases upon increased cell density and entrance to stationary phase in high-oxygen-tension cultures, the oxygen levels in the shift experiments were much lower by design. Consequently, induction of the *bop* gene cluster in strain II-7 occurs if the oxygen tension is low enough, demonstrating that the *bop* gene cluster is not completely uncoupled from regulation by oxygen tension in this strain.

In Fig. 1B, we propose a model based on data obtained from wild-type cultures grown under high oxygen tension in the light and in the dark. Our proposal that the putative *brp* protein may be a membrane-bound light sensor is based in part on the dramatic induction of *brp* transcription by light

and the apparent lack of induction by the somewhat lower oxygen tension present during stationary phase in wild-type cultures grown under high oxygen tension in the dark. However, *brp* transcription was induced in cultures of strain II-7 shifted to low oxygen in the dark. It is possible that the induction of *brp* transcription seen in shifted cultures of II-7 in the dark might be a direct response either to the significantly lower oxygen tension or to an elevated level of a regulatory protein which is induced under these conditions. Indeed, under these conditions maximum *bat* transcript levels are threefold higher than the highest levels achieved in high-oxygen-tension cultures in the dark. Our hypothesis that the putative *brp* protein is a membrane-bound light sensor and/or transmitter remains a possibility, since under all conditions tested *brp* transcription is significantly more sensitive to the presence of light than to changes in oxygen tension.

Preliminary observations (25) indicate that genes involved in the synthesis of gas vacuole proteins, retinal, and bacterioruberin may also be regulated by low oxygen tension and high light intensity. Together with the *bop* gene cluster, these genes may comprise a multigene family activated by common environmental parameters (i.e., a stimulon; 18), similar to genes in other systems which are activated by starvation in the stationary phase of growth (16) or by heat shock (19).

ACKNOWLEDGMENTS

We thank Herbert Boyer for support; Felix and Roswitha Gropp for helpful discussions; Mark Neuman of Lab-Line for donating the environmental shaker, which was expertly modified by Douglas McVay and Michael O'Grady to form the phototrophic chamber; and Bob Moody for helpful discussions about light.

This work was supported by Public Health Service grant GM31785 from the National Institute of General Medical Sciences and National Research Service Award 5T32CA09043 to R.F.S.

REFERENCES

1. **Betlach, M., J. Friedman, H. W. Boyer, and F. Pfeifer.** 1984. Characterization of a halobacterial gene affecting bacterio-opsin gene expression. Nucleic Acids Res. **12**:7949–7959.
2. **Betlach, M., F. Pfeifer, J. Friedman, and H. W. Boyer.** 1983. Bacterio-opsin mutants of *Halobacterium halobium*. Proc. Natl. Acad. Sci. USA **80**:1416–1420.
3. **Betlach, M. C., R. F. Shand, and D. M. Leong.** 1989. Regulation of the bacterio-opsin gene of a halophilic archaebacterium. Can. J. Microbiol. **35**:134–140.
4. **Braiman, M., J. Bubis, T. Doi, H. B. Chen, S. L. Flitsch, R. R. Franke, M. A. Gilles-Gonzalez, R. M. Graham, S. S. Karnik, H. G. Khorana, B. E. Knox, M. P. Krebs, T. Marti, T. Mogi, T. Nakayama, D. D. Oprian, K. L. Puckett, T. P. Sakmar, L. J. Stern, S. Subramaniam, and D. A. Thompson.** 1988. Studies on light transduction by bacteriorhodopsin and rhodopsin. Cold Spring Harbor Symp. Quant. Biol. **53**:355–364.
5. **Danon, A., and W. Stoeckenius.** 1974. Photophosphorylation in *Halobacterium halobium*. Proc. Natl. Acad. Sci. USA **71**:1234–1238.
6. **DasSarma, S., U. L. RajBhandary, and H. G. Khorana.** 1983. High-frequency spontaneous mutation in the bacterio-opsin gene in *Halobacterium halobium* is mediated by transposable elements. Proc. Natl. Acad. Sci. USA **80**:2201–2205.
7. **Drummond, M. H., and J. C. Wootton.** 1987. Sequence of *nifL* from *Klebsiella pneumoniae*: mode of action and relationship to two families of regulatory proteins. Mol. Microbiol. **1**:37–44.
8. **Dunn, R., J. McCoy, M. Simsek, A. Majumdar, S. Chang, U. RajBhandary, and H. G. Khorana.** 1981. The bacteriorhodopsin gene. Proc. Natl. Acad. Sci. USA **78**:6744–6748.
9. **Gropp, F., and M. Betlach.** Unpublished data.
10. **Gropp, R., and M. Betlach.** Unpublished data.
11. **Hartmann, R., and D. Oesterhelt.** 1977. Bacteriorhodopsin-mediated photophosphorylation in *Halobacterium halobium*. Eur. J. Biochem. **77**:325–335.
12. **Henderson, R., J. M. Baldwin, T. A. Ceska, F. Zemlin, E. Beckmann, and K. H. Downing.** 1990. A model for the structure of bacteriorhodopsin based on high resolution electron cryomicroscopy. J. Mol. Biol. **213**:899–929.
13. **Leong, D., H. Boyer, and M. Betlach.** 1988. Transcription of genes involved in bacterio-opsin gene expression in mutants of a halophilic archaebacterium. J. Bacteriol. **170**:4910–4915.
14. **Leong, D., F. Pfeifer, H. Boyer, and M. Betlach.** 1988. Characterization of a second gene involved in bacterio-opsin gene expression in a halophilic archaebacterium. J. Bacteriol. **170**: 4903–4909.
15. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual, p. 190. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
16. **Matin, A., E. A. Auger, P. H. Blum, and J. E. Schultz.** 1989. Genetic basis of starvation survival in non-differentiating bacteria. Annu. Rev. Microbiol. **43**:293–316.
17. **Melton, D., P. Krieg, M. Rebagliati, T. Maniatis, K. Zinn, and M. Green.** 1984. Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucleic Acids Res. **12**:7035–7056.
18. **Neidhardt, F., J. Ingraham, and M. Schaechter.** 1990. Physiology of the bacterial cell, p. 382–383. Sinauer Associates, Inc., Sunderland, Mass.
19. **Neidhardt, F. C., and R. A. VanBogelen.** 1987. Heat shock response, p. 1334–1345. *In* J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, vol. 2. American Society for Microbiology, Washington, D.C.
20. **Oesterhelt, D., and G. Krippahl.** 1983. Phototrophic growth of halobacteria and its use for isolation of photosynthetically-deficient mutants. Ann. Microbiol. Inst. Pasteur **134 B**:137–150.
21. **Oesterhelt, D., and W. Stoeckenius.** 1971. Rhodopsin-like protein from the purple membrane of *Halobacterium halobium*. Nature (London) New Biol. **233**:149–152.
22. **Oesterhelt, D., and W. Stoeckenius.** 1973. Functions of a new photoreceptor membrane. Proc. Natl. Acad. Sci. USA **70**:2853–2857.
23. **Pfeifer, F. A., H. W. Boyer, and M. C. Betlach.** 1985. Restoration of bacterio-opsin gene expression in a revertant of *Halobacterium halobium*. J. Bacteriol. **164**:414–420.
24. **Rehorek, M., and M. P. Heyn.** 1979. Binding of all-trans-retinal to the purple membrane. Evidence for cooperativity and determination of the extinction coefficient. Biochemistry **18**:4977–4983.
25. **Shand, R. F., and M. C. Betlach.** Unpublished data.
26. **Sumper, M., and G. Herrmann.** 1976. Biosynthesis of purple membrane: regulation of bacterio-opsin synthesis. FEBS Lett. **69**:149–152.
27. **Sumper, M., and G. Herrmann.** 1976. Biosynthesis of purple membrane: control of retinal synthesis by bacterio-opsin. FEBS Lett. **71**:333–336.
28. **Sumper, M., H. Reitmeier, and D. Oesterheldt.** 1976. Biosynthesis of the purple membrane of halobacteria. Angew. Chem. Ed. Engl. **15**:187–194.
29. **Yang, C., and S. DasSarma.** 1990. Transcriptional induction of purple membrane and gas vesicle synthesis in the archaebacterium *Halobacterium halobium* is blocked by a DNA gyrase inhibitor. J. Bacteriol. **172**:4118–4121.