Aerobic Repression of the *Rhodobacter capsulatus* *bchC* Promoter Involves Cooperative Interactions between CrtJ Bound to Neighboring Palindromes*

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Previous studies demonstrated that bacteriochlorophyll, carotenoid, and light harvesting gene expression in *Rhodobacter capsulatus* is repressed under aerobic growth conditions by the repressor CrtJ. Isolated CrtJ is known to bind to the palindrome TGTN12ACA, which is present in two copies in the *bchC* promoter, one of which spans the −35 and the other the −10 σ-70 recognition sequences. In this study, we demonstrate that CrtJ binds to the two palindromic sites in the *bchC* promoter in a cooperative manner. The level of cooperativity of CrtJ binding to the −35 palindrome was shown to be 26-fold. A distance of 8 base pairs between the two palindromic sites was shown to be critical for cooperative binding, as evidenced by the disruption of binding that resulted when +6 and +11 base pairs were inserted between the palindromes.

The purple nonsulfur photosynthetic bacterium *Rhodobacter capsulatus* synthesizes a photosystem under anaerobic growth conditions. Regulation of photosystem synthesis is primarily mediated by controlling the initiation of photosynthesis gene expression (reviewed in Refs. 1 and 2). In a previous study, we demonstrated that aerobic repression of bacteriochlorophyll (*bch*), carotenoid (*crt*), and light harvesting II (*puc*) gene expression involves the transcription factor CrtJ (3, 4). Biochemical analysis of purified CrtJ indicates that it functions as a redox-sensitive DNA-binding protein that optimally binds to the *bchC* promoter under oxidizing conditions (4).

DNase I footprint titration assays of CrtJ binding to the *bchC* promoter region indicates that CrtJ protects two conserved palindromes (TGTN12ACA), one of which spans the −35 and the other the −10 σ-70 sequence recognition motifs (3). Hill coefficient determination from DNA binding isotherms indicate that CrtJ binds to the *bchC* promoter region as a tetramer, presumably with CrtJ monomers interacting with each palindrome half site (4).

Inspection of promoters that are regulated by CrtJ indicates that there are two motifs (Fig. 1). One class, represented by the *bchC* promoters of *R. capsulatus* and *Rhodobacter sphaeroides* and by the *R. sphaeroides* *puc* promoter, contains two CrtJ recognition palindromes, one of which spans the −35 and the other the −10 σ-70 recognition sequences (4–8). The other class, represented by the *R. capsulatus* *puc* and *crtI* promoters and by the *R. sphaeroides* *crtI* promoter, has only a single CrtJ palindrome that is near or overlapping with the −10 or −35 regions (9, 10, 11). It is not clear why some promoters contain only one palindrome and others contain two. More detailed analysis of these promoters is needed to obtain a better understanding of aerobic repression of photosystem gene expression by CrtJ.

In the present study, we utilized a combination of gel mobility shift and DNase I footprint analyses to demonstrate that CrtJ binding to the two palindromic sites in the *bchC* promoter region occurs in a cooperative fashion. DNA binding isotherms indicate that there is a 26-fold cooperativity of CrtJ binding to the −35 palindrome. The spacing of 8 base pairs (bp) between the two *bchC* palindromes is also shown to be critical for CrtJ binding. In a companion study (10), we demonstrate that aerobic repression of the *puc* and *crtI* promoters involves cooperative interactions among CrtJ bound to distant palindromes.

**EXPERIMENTAL PROCEDURES**

**Strains, Media, and Growth Conditions—*Escherichia coli* strain NM522 was used for routine cloning procedures (12). For overexpression of CrtJ, strain BL21(DE3) was utilized (13). Plasmids pDAY230 (14), pUC18 (15), and pET28-CrtJ (4) have been described previously. Luria broth was used for agar-solidified plates and for liquid cultures (16). Ampicillin and kanamycin were used at 100 μg/ml and 30 μg/ml, respectively.

**Heterologous Expression and Isolation of CrtJ—His-tagged CrtJ was heterologously overexpressed in *E. coli* using a T7 RNA polymerase-based overexpression system as described previously (4, 13). Purification of His₆-promoted CrtJ from a 100-ml culture of cells was performed using nickel column affinity chromatography with all purification steps performed at 4 °C (Novagen). Fractions containing the highest CrtJ concentrations were pooled and dialyzed overnight in 1 liter of buffer composed of 50 mM Tris-HCl, pH 7.9, 50 mM potassium acetate, 1 mM EDTA, and 20% glycerol. The purified protein was then partitioned into 30-μl aliquots, subjected to rapid freezing using a dry ice-ethanol mixture, and stored at −80 °C. Protein concentrations were determined using the Bradford assay (17) (Bio-Rad). The percent active fraction of CrtJ was determined with a nitrocellulose filter binding assay according to the method of Withereil and Uhlenbeck (18) and Ponnampalam and Bauer (4).

**Plasmid Construction—**Several plasmids were constructed to separately clone the upstream (−30 to −47) and downstream palindromes (−4 to −21) from the *bchC* promoter region, as well as to introduce +6 and +11-bp insertions between the upstream and downstream palindromes. For these constructions, several segments of the *bchC* promoter region were generated by polymerase chain reaction (PCR) amplification and then cloned into appropriate cloning vectors. The individual PCRs contained 10 mM Tricine buffer (19), 0.3 mM dATP, 0.3 mM dGTP, 0.3 mM dTTP, 0.3 mM dCTP, 100 μmol of each primer, and 12.5 ng of DNA template.

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†† The abbreviations used are: bp, base pair(s); PCR, polymerase chain reaction; Tricine, N,N-di-hydroxy-1,1-bis(hydroxymethyl)ethylglycine; TBE, Tris-borate EDTA.
BamHI-EcoRI-digested pDay23I template DNA (14). The PCR cycle (PTC-100 Programmable Thermal Controller, MJ Research, Inc.) was initiated with a "hot start," which involved preincubation of the PCR at 98 °C for 2.5 min followed by a reduction of reaction temperature to 90 °C, at which point, 1 unit of Taq DNA polymerase was added to the reaction buffer. Amplification was performed using 40 cycles of 20 s at 97 °C, 30 s at 62 °C, and 2 min at 72 °C. The reactions were then finished with a 20-min incubation period at 65 °C, and the resulting fragments were cloned into the TA cloning vector pCRII (Invitrogen).

To PCR amplify a 67-bp fragment containing the upstream palindrome, primers BchC3 (5′-GGGAGATCCGGACCTTAATGGTGACCC-3′) and BchC5 (5′-GGGATCCGGACCTTAATGGTGACCC-3′) were used. For PCR amplification of 110-bp fragment containing the downstream palindrome, primers BchC2 (5′-CCGGATCCGGACCTTAATGGTGACCC-3′) and BchC4 (5′-CCGGATCCGGACCTTAATGGTGACCC-3′) were used. A 114-bp fragment, which also contains the downstream palindrome, was amplified using BchC2 in conjunction with primer BchC6 (5′-CCGGATCCGGACCTTAATGGTGACCC-3′). Each of the PCR products was cloned into the TA tail site of pCRII (Invitrogen). Note that to facilitate subsequent subcloning steps, BamHI sites were engineered at the 5′-ends of oligonucleotides BchC4, BchC5, and BchC6 (underlined bases).

Construction of +6- and +11-bp insertions between the palindromes involved first subcloning the amplified upstream palindrome from the vector pCRII as a 90-bp BamHI-EcoRI restriction fragment (the BamHI site came from the primer, and the EcoRI site came from the vector) into the vector pUC18 to construct the plasmid pUC3.5. For introducing a +11-bp insertion between the palindromes, we next subcloned the BchC2/BchC4 PCR-generated downstream palindrome fragment into pUC3.5. This was accomplished by subcloning a 146-bp BamHI-Xhol fragment from pCRII (the BamHI site was present in the primer, whereas the Xhol site was present in the vector) into the BamHI-SalI site of pUC3.5 vector, resulting in the vector pUC3.5+2.4. For constructing a +11-bp insertion between the palindromes, we subcloned the 151-bp BchC2/BchC6 PCR-generated downstream palindrome fragment from pCRII into pUC3.5 in a manner analogous to that described for the +6 insertion, generating the recombinant pUC3.5+2.6. Each of the final constructs were then sequenced to confirm the presence of +6 and +11 insertions between the palindromes.

**Results and Discussion**

**CrtJ Demonstrates Cooperative Binding to Two Palindromic Sites in the bchC Promoter**—We assayed for cooperative interactions between CrtJ bound to the −35 and −10 palindromes in the bchC promoter by performing gel mobility shift and DNase I footprint analysis with a variety of DNA probes. Specific probes that were used included (i) a probe that contained the wild type promoter region flanked by 58 and 79 bp of upstream and downstream DNA, respectively (Fig. 2A); (ii) a segment that contains only the −35 palindrome flanked by 58 and 134 bp of DNA (Fig. 2B); and (iii) a segment that contains...
only the −10 palindrome flanked by 168 and 79 bp of DNA (Fig. 2C).

Similar to what we reported in a previous study (4), we observed that the entire wild type \textit{bchC} promoter probe exhibited a mobility shift when incubated with 0.25 and 0.50 μg of isolated CrtJ (Fig. 3, lanes 2 and 3). In contrast, there was no observable mobility shift using probes that only contain the −35 palindrome (Fig. 3, lanes 5–6) or only the −10 palindrome (Fig. 3, lanes 8–9) when using similar amounts of CrtJ. The absence of a discrete gel shift with probes containing only the −35 or −10 palindromes gives a qualitative indication that there is cooperative binding of CrtJ at the two palindromic sites in the wild type probe.

DNase I footprint titration analyses were subsequently performed on the same probes used for gel mobility shift assays to quantitatively address cooperative interactions between CrtJ bound to the neighboring palindromes. As shown in Fig. 4 (left panel), footprint analysis with the wild type probe exhibited protection of both palindromes at a CrtJ level as low as 0.125 μg. In contrast, the probe that contains only the −35 palindrome (Fig. 4, middle panel) required approximately 2.0 μg of CrtJ to visualize protection of the palindromic region. No protection was observed on the −10 palindrome (Fig. 4, right panel) even at the highest level of CrtJ that was used (16 μg). In addition to providing additional evidence of cooperativity among CrtJ bound to the two palindromes, the footprint results also indicate that CrtJ has a higher affinity for the −35 palindrome than for the −10 palindrome.

Isotherms of CrtJ binding to the −35 palindrome were generated by quantitating fractional protection of the −35 palindrome to DNase I digestion using a PhosphorImager as described previously (4, 21). Values of fractional protection plotted against the logarithm of protein concentration (Fig. 5) produced curves with 26-fold different midpoint values of CrtJ protection of the −35 palindrome when in the presence or absence of the −10 palindrome. When corrected for percentage of active protein in the isolated CrtJ preparation (see under “Experimental Procedures” and Ref. 4), we calculate an EC$_{50}$ value of 7.8 × 10$^{-9}$ M for the −35 palindrome alone versus 0.3 × 10$^{-9}$ M for the −35 palindrome in the presence of the −10 palindrome.

The Distance of 8 Base Pairs Separating the −35 and −10 Palindromes in the \textit{bchC} Promoter Region Is Critical for CrtJ Binding—We next addressed whether the 8-bp spacing be-
between the −35 and −10 palindromes is critical for cooperative interactions. For this analysis, we constructed probes in which the intervening sequences between the two bchC palindromes were increased from 8 to 14 bp (half-helical insertion) or from 8 to 19 bp (full-helical insertion) (Fig. 2, D and E, respectively).

The results of gel mobility shift analysis with these probes indicate that increasing the spacing between the palindromes to 14 or 19 bp abolishes the formation of a stable DNA-CrtJ complex (Fig. 6, lanes 6–8 and 10–12, respectively). DNase I footprint titration analyses on these probes gave results similar to those observed with the half sites, namely, partial protection of the −35 palindrome at high input levels of CrtJ (>2 µg) and...
no protection of the −10 palindrome (data not shown). This indicates that the 8-bp spacing between the two palindromic sites is indeed important for proper cooperative binding of CrtJ to the \( bchC \) promoters.

In conclusion, we have shown by both gel mobility shift and footprint analyses that CrtJ binds in a cooperative fashion to the two palindromic sites of the \( bchC \) promoter region. CrtJ binding to the −35 palindrome shows 26-fold cooperativity when spaced 8 nucleotides apart from the −10 palindrome. This is of the same order of magnitude of cooperative DNA binding that has been observed for other proteins. For example, in the \( P_\lambda \) promoter of bacteriophage lambda, cooperativity increases affinity of the \( \lambda \) repressor to the middle operator site (OR2) by 25-fold and to the right (OR1) operator site by 2-fold (22, 23, 24). CrtJ binding to the class of photosynthesis promoters that contain two closely spaced (7–8 bp) palindromes thus follows that of other well studied DNA-binding proteins that utilize cooperativity to facilitate binding.

In the companion report by Elsen et al. (10), we have investigated the nature of CrtJ repression of photosynthesis promoters that contain only a single palindrome in the −10 to −35 promoter region. The Elsen et al. (10) study demonstrates that repression of this class of promoters requires the presence of a second distant (76–240 bp) palindrome. Thus, effective repression of target promoters by CrtJ requires cooperative interactions between CrtJ bound to two palindromes in each of the promoter regions that have been studied.

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