Autorepression of RctB, an Initiator of *Vibrio cholerae* Chromosome II Replication†

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The RctB protein binds to the origin of replication of *Vibrio cholerae* chromosome II (chrII) and is required for oriCIIVc-based replication. Here, we found that RctB acts as an autorepressor, inhibiting rctB transcription. Integration host factor promotes rctB transcription, while Dam and DnaA, factors required for replication of both *V. cholerae* chromosomes, influence RctB autorepression. Thus, RctB appears to regulate chrII replication as both an initiator and a transcription repressor, and its synthesis is modulated by factors that govern replication of both chromosomes.

The genome of *Vibrio cholerae*, the causative agent of the severe diarrheal disease cholera, is divided unequally between two circular chromosomes. Most if not all of the many species that constitute the family *Vibrionaceae* have similarly divided genomes (12, 17–19). As more bacterial genomes have been investigated, it has become clear that multipartite genomes are not uncommon and are found among diverse prokaryotic phyla (2). Almost all studies of bacterial chromosome replication and segregation have utilized organisms with a single chromosome, yet the models derived from these studies may not fully apply to bacteria with multipartite genomes (2).

We previously constructed minichromosomes to identify the minimal replicons of the two *V. cholerae* chromosomes (4). We found that oriCIIVc, the origin of replication of the larger chromosome I (chrI), is similar in sequence to oriC, the well-characterized origin of replication of the *Escherichia coli* chromosome. Like oriC, oriCIIVc includes five DnaA boxes (binding sites for the DnaA initiator protein), a putative binding site for integration host factor (IHF, a histone-like protein that binds DNA), several sites for methylation by DNA adenine methyltransferase (Dam, which is involved in regulating timing of replication in *E. coli*), and an AT-rich region (where strand opening is believed to originate).

The origin of replication of chrII, oriCHVc, does not resemble oriCIIVc in terms of sequence identity (4). However, it contains some features common to many bacterial origins of replication, such as one DnaA box, a binding site for IHF, several sites for Dam methylation, and an AT-rich region. In addition to these features, oriCHVc contains a 12-base-pair repeat that is required for oriCHVc-based replication. While the intergenic region containing oriCHVc can replicate autonomously in *E. coli*, oriCHVc-based replication requires two novel *V. cholerae* genes which flank oriCHVc (Fig. 1). One of these genes, rctB, encodes an origin-binding protein and is conserved among diverse genera of the family *Vibrionaceae*. The other gene, rctA, codes for an untranslated RNA and not a protein.

The distinct replication requirements for the two *V. cholerae* chromosomes suggest that initiation of chrII replication may, at least in part, be controlled independently from chrI. However, our studies of the kinetics of *V. cholerae* chromosome replication revealed that the two chromosomes initiate replication in a coordinated, synchronous manner (3). We found that oriCIIVc and oriCHVc-based replication share requirements for certain factors that have been implicated in the regulation of bacterial chromosome replication in other organisms, such as DnaA and Dam methyltransferase (4). These shared replication factors may help mediate coordinated replication of the chromosomes. However, our understanding of the interplay of the various *V. cholerae* replication factors and how they act to coordinate chromosome replication is limited.

Since the *V. cholerae* RctB protein binds specifically to oriCIIVc and is required only for oriCIIVc-based replication, RctB appears to be a chrII-specific replication initiator. Here, we identified the rctB promoter and investigated its regulation. We found that RctB is autoregulatory and represses its own transcription. IHF enhances rctB transcription, and Dam and DnaA influence RctB-mediated repression of rctB transcription. In addition to its own promoter, RctB also regulates the promoter for rctA.

To identify the rctB transcriptional start site, we isolated RNA from *V. cholerae* strain N16961 and used 5′ rapid amplification of cDNA ends (Invitrogen). This analysis revealed a single putative transcriptional start site 40 bp upstream of the rctB start codon (Fig. 1; see also Fig. S1 in the supplemental material). A 209-bp fragment spanning this site could drive transcription from a promoterless lacZ gene (see below), suggesting that this region harbors the rctB promoter, P_rctB.

BPROM, a computer program designed to identify sigma 70-dependent promoters for bacterial genes (SoftBerry, Mount Kisco, NY), predicted −10 and −35 sites upstream of the rctB transcriptional start site (see Fig. S1 in the supplemental material). Experimental verification that the predicted −10 and −35 sequences are required for activity of P_rctB has recently been provided by another lab (14).

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We explored the influence of sequences upstream of \( P_{rctB} \) on \( rctB \) expression by studying the activity of \( P_{rctB} \) using a set of \( rctB \) transcriptional reporter plasmids. DNA fragments of various lengths from the intergenic region (\( \text{ig}2 \)) upstream of the \( rctB \) open reading frame were introduced upstream of a promoterless \( \lambda \)-galactosidase (\( \beta \)-galactosidase) gene. The smallest fragment, found in the B1 reporter, started at base pair 924 (numbers are according to the TIGR annotation of the N16961 genome) and included 209 bp upstream of the \( rctB \) start codon; the largest fragment, in the B3 reporter, included the entire \( \text{ig}2 \) intergenic region between \( rctB \) and the divergently transcribed \( rctA \). Each of these reporters exhibited significant \( \beta \)-galactosidase activity in \( E. coli \), indicating that there are no \( V. cholerae \)-specific factors required for \( P_{rctB} \) function. Also, the amounts of \( \beta \)-galactosidase activity of all three reporters were very similar (Fig. 1), suggesting that there is only one rightward-facing promoter within \( \text{ig}2 \) upstream of \( lacZ \) and that there are no key cis-acting sites in \( \text{ig}2 \) upstream of base pair 924 that control \( P_{rctB} \) expression. In \( V. cholerae \), the level of \( \beta \)-galactosidase activity generated by these reporters was \( \sim 3 \)-fold lower than in \( E. coli \), suggesting that there is a \( V. cholerae \)-specific factor(s) that represses \( P_{rctB} \) activity (Fig. 1). As in \( E. coli \), the activities of the B1 and B2 reporters in \( V. cholerae \) were similar, providing further support for the idea that sequences upstream of base pair 924 do not control \( P_{rctB} \) expression. The B3 reporter contains \( inc \) sequences that negatively regulate chrII replication (4), and as expected, we were unable to introduce this reporter into \( V. cholerae \).

We speculated that RctB autorepression might account for the reduced activity of \( P_{rctB} \) in \( V. cholerae \) compared to \( E. coli \) for two reasons. First, we previously showed that RctB binds to oriC\( _{VC} \) in the region now known to contain \( P_{rctB} \) (4), and second, initiator proteins are commonly autoregulated (1, 10). This hypothesis proved correct; the expression of all three \( P_{rctB} \) reporters was repressed by RctB more than fivefold when RctB

![FIG. 1. Reduced \( \beta \)-galactosidase activity of \( rctB::\lambda\)-lacZ reporters in \( V. cholerae \) versus \( E. coli \).](http://jb.asm.org/)

![FIG. 2. RctB repression of \( rctB \) and \( rctA \) transcription.](http://jb.asm.org/)
was overproduced from a plasmid in *E. coli* (Fig. 2). RctB binds to multiple sites in ig2 (4); however, there was no obvious correlation between the degree of RctB-mediated repression of the three *rctB* reporters and the number of RctB binding sites in the individual reporters, suggesting that potential interactions between different RctB molecules bound throughout ig2 do not influence \( \text{P}_{\text{rctB}} \) activity. As in *E. coli*, when *rctB* was overexpressed in *V. cholerae*, \( \text{P}_{\text{rctB}} \) activity was also reduced (data not shown). Overexpression of RctB did not repress the activity of a control reporter, indicating that RctB repression of \( \text{P}_{\text{rctB}} \) is specific (Fig. 2). Thus, like both DnaA and Rep proteins of iteron plasmids, RctB appears to be an autoregulatory initiator protein that represses its own synthesis.

To study whether *rctB* transcription is influenced by other factors that are required for chrII replication, such as Dam methyltransferase and DnaA, we measured \( \text{P}_{\text{rctB}} \) activity from the B1 and B3 *rctB* reporters in *E. coli* strains harboring mutations in *dam* or *dnaA*. Dam methylation is known to regulate gene expression (7), and there are several potential sites for methylation by Dam methyltransferase in the vicinity of \( \text{P}_{\text{rctB}} \) (Fig. 1). The \( \beta \)-galactosidase activities of the two *rctB* reporters were similar in wild-type and *dam* *E. coli* strains, indicating that Dam methylation does not significantly influence \( \text{P}_{\text{rctB}} \) activity (Fig. 3A). However, Dam methylation appears to enhance RctB autorepression. In *dam* *E. coli*, overexpression of *rctB* resulted in somewhat less repression of the *rctB* reporters than was observed in wild-type *E. coli*; this effect was strongest for reporter B1 (Fig. 3A). These findings may suggest that methylation increases the affinity of RctB for its promoter.

DnaA is known to act as a transcription regulator as well as an initiator of DNA replication (11). Since \( \text{P}_{\text{rctB}} \) is close to the single DnaA box in *oriC* (Fig. 1), we tested whether DnaA influences \( \text{P}_{\text{rctB}} \) activity by comparing the levels of \( \text{lacZ} \) expression from the B3 and B1 *rctB* reporters, one of which (B3)
includes the DnaA box and the other of which does not (Fig. 1). The β-galactosidase activities of these two rctB reporters were similar (Fig. 1), suggesting that DnaA binding to ig2 does not influence P_{rctB} promoter activity. Consistent with this idea, we found that the β-galactosidase activities of these two reporters were similar in a dnaA deletion strain (with an integrated R1 ori [5]) and isogenic wild-type E. coli (Fig. 3B). However, the degrees of RctB autorepression were different in these strains (Fig. 3B). Although in both backgrounds RctB repressed its own synthesis, in the absence of DnaA, RctB-mediated repression was enhanced 2.8-fold for reporter B3 and less so for B1. This may suggest that DnaA antagonizes RctB binding to P_{rctB}.

The nucleoid-associated factor IHF is a heterodimer consisting of HimA and HimB and is known to modulate transcription of some bacterial genes (9). We investigated whether IHF influences P_{rctB} activity. oriCI_{Vc} contains a DNA sequence that is similar to the E. coli IHF consensus binding site (4), and we have found that the E. coli IHF protein binds to the region of oriCI_{Vc} containing this site (our unpublished observations). IHF appears to activate transcription from P_{rctB} as transcription from P_{rctA} was reduced ~2-fold in an E. coli himA mutant strain compared with wild-type E. coli (Fig. 3C). The similar reductions in the β-galactosidase activities of the B1 and B2 reporters in himA versus wild-type E. coli suggest that the activating effect of IHF requires only, at the most, the sequence present in the small B1 reporter. The activity of a control reporter was not reduced in himA E. coli, indicating that the effect of the himA deletion is not due to a global defect in transcription levels. In V. cholerae, IHF activation of transcription from P_{rctB} was even more pronounced, as the β-galactosidase activities of the B1 and B2 reporters were four to five times lower in himA than in wild-type V. cholerae. These observations suggest that IHF contributes to efficient P_{rctB} activity, as is the case for several other promoters where IHF promotes architectural changes in the DNA. Alternatively, IHF could also activate transcription by directly contacting RNA polymerase (9). IHF does not appear to influence the binding of RctB to P_{rctB}, since the reporters were repressed to similar extents by RctB in himA and wild-type E. coli (Fig. 3C).

Since RctB binds to the left side of ig2 just upstream of rctA (4), we tested whether RctB regulates rctA transcription. Using BPROM, we identified a putative rctA promoter (P_{rctA}) approximately 80 bp upstream of the annotated gene (Fig. 1). This region of DNA can drive lacZ transcription, since a P_{rctA}:lacZ reporter (A1) yielded ~93 Miller units of β-galactosidase activity in E. coli (Fig. 1). Introduction of a 3-bp substitution mutation into the putative −10 sequence of P_{rctA} in A1 decreased β-galactosidase activity ~10-fold, indicating that P_{rctA} is indeed a true promoter. RctB overproduction reduced the β-galactosidase activity from A1 more than eight-fold, indicating that P_{rctA} is repressed by RctB (Fig. 2). Thus, RctB represses at least two genes, rctA and rctB, required for chrII replication.

Our findings reveal that RctB is a multifunctional protein. Previously, we found that RctB is required for oriCI_{Vc}-based replication and that RctB binds to several sites in ig2, suggesting that RctB functions as a chrII-specific replication initiator. This idea is supported by the recent observation that RctB levels determine the copy number of an oriCI_{Vc}-based minichromosome in E. coli (14). Besides acting as a chrII-specific initiator, RctB also functions as a transcriptional repressor, inhibiting transcription from P_{rctB} and from P_{rctA}. Thus, RctB controls oriCI_{Vc}-based replication on at least three levels: as an initiator of replication, as an autorepressor, and as a repressor of rctA.

RctB binds in the vicinity of P_{rctB}, and therefore it is likely that RctB directly represses its own promoter. RctB autorepression, which may be a critical determinant of RctB levels and chrII replication, appears to be influenced by DnaA and Dam, two host factors that are essential for replication of both V. cholerae chromosomes (4). Dam modestly potentiates RctB autorepression, probably by altering RctB binding to sites that influence P_{rctB} activity. DnaA modestly inhibits RctB autorepression, perhaps by interacting with RctB and inhibiting its binding to P_{rctB}. Interactions between DnaA and plasmid Rep proteins have been described previously (8). Thus, there appears to be a complex interplay of factors essential for chrI and chrII replication that, along with RctB, govern rctB transcription. Understanding the molecular details of RctB-DNA and RctB-protein interactions will enhance our understanding of how the two chromosomes initiate replication in synchrony.

IHF promotes transcription from P_{rctB} but does not influence RctB binding to this region. The putative IHF binding site is 129 bp from the start site of rctB transcription, and the region upstream of the IHF binding site is relatively AT rich. This arrangement of an AT-rich region and IHF site upstream of a promoter is similar to that of the ilvP_G promoter in E. coli, which is also activated by IHF (13). IHF activates ilvP_G by translocating superhelical energy; binding to its cognate site prevents destabilization of the upstream AT-rich region while promoting duplex destabilization of the −10 region of the promoter (15). Destabilization is correlated with open complex formation as well as increased transcriptional activity of ilvP_G. It is tempting to speculate that IHF activates P_{rctB} by a similar mechanism.

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