ATP negatively regulates the initiator protein of *Vibrio cholerae* chromosome II replication

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***Vibrio cholerae*, the agent of cholera, has two circular chromosomes. In bacteria that contain a single chromosome, initiation of chromosome DNA replication is mediated by DnaA, a AAA+ ATPase that unwinds the origin of replication. There is little knowledge regarding initiation of chromosome replication in bacteria with more than one chromosome. Here, we purified *V. cholerae* DnaA and RctB, which have been implicated in the replication of *V. cholerae* chromosome II, and characterized their activities *in vitro*. We found that RctB has origin-specific unwinding activity and can melt the origin of chromosome II (*oriCIIvc*) but not the origin of chromosome I (*oriCIvc*); conversely, DnaA promoted the unwinding of *oriCIvc* and not *oriCIIvc*. The activity of DnaA and several plasmid initiator proteins is stimulated by ATP binding. We found that RctB bound and hydrolyzed ATP even though RctB lacks any apparent ATP-binding motifs. However, we unexpectedly found that ATP inhibited the *oriCIIvc* binding activity of RctB, suggesting that the ATP-bound form of RctB cannot initiate replication of chromosome II. Supporting this idea, we identified an RctB mutant that does not bind ATP and found that expression of this ATP-blind RctB mutant in *V. cholerae* leads to significant overinitiation of chromosome II and marked inhibition of *V. cholerae* growth. These observations suggest that the rules that license the replication of the two *V. cholerae* chromosomes differ.**

initiation of replication | RctB | DnaA | cell cycle

Bacterial chromosome replication is initiated by the binding of an "initiator" protein to the origin of replication. DnaA, a conserved AAA+ ATPase, is thought to be the initiator of chromosome DNA replication in nearly all eubacteria (1). Most of our knowledge of DnaA function and regulation has been derived from studies of *Escherichia coli*. In this microorganism, DnaA binds to several 9-bp sequences (DnaA boxes) found in *oriC* (2), the origin of replication, leading to the formation of a nucleoprotein complex. Formation of this DnaA–*oriC* complex triggers the melting of a thermodynamically unstable A+T-rich sequence within *oriC*, known as the DUE (DNA Unwind Element). After unwinding of the DUE, DnaA directs loading of the DnaB helicase onto the ssDNA along with DnaC, the helicase loader (3). At this point, other components of the replisome load on to *oriC* and rapid bidirectional replication of the chromosome proceeds.

Only the ATP bound form of DnaA is active in initiation (4). It appears that binding of ATP to DnaA contributes to several aspects of initiation, although the underlying mechanisms are not fully understood. First, DnaA complexed with ATP has a higher affinity for certain DnaA binding sites in *oriC* (5). Second, it is thought that ATP-dependent interactions between DnaA molecules bound to different sites within *oriC* enable the formation of a higher-ordered nucleoprotein complex that is capable of melting the DUE (6). Although DnaA is an ATPase, the energy derived from ATP hydrolysis is not required for unwinding the DUE. Instead, the ATPase activity of DnaA is thought to be important for regulating its activity. Consistent with this idea, *E. coli* expressing an ATPase-deficient form of DnaA displays an overinitiation phenotype (7).

Not all bacteria contain a single circular chromosome like *E. coli*, and DnaA may not function as the initiator of replication of secondary chromosomes in such species. There is already evidence that DnaA does not function as the initiator of replication of the second chromosome in the Vibrionaceae and Photobacteriacea. The genomes in the dozens of species in these families, which include several important human and fish pathogens, all consist of two circular chromosomes (8). The origin of replication of each of the chromosomes in *Vibrio cholerae*, the cause of the severe diarrheal disease cholera, has been defined and characterized (9). *V. cholerae* chromosome I (chrI) is larger than chromosome II (chrII) and its origin of replication, *oriCIvc*, is similar in sequence and features to *oriC*. Like *oriC*, *oriCIvc* contains eight DnaA boxes, an AT-rich sequence, and a binding site for IHF. Moreover, replication of an *oriCIvc*-dependent minichromosome requires DnaA (9), suggesting that the process for initiating replication at *oriCIvc* resembles initiation at *oriC*. Finally, overexpression of DnaA leads to overinitiation of chrI but not chrII (10), suggesting that DnaA specifically triggers replication at *oriCI*.

The origin of replication of chrII, *oriCIIvc*, does not exhibit significant sequence similarity to *oriCIvc* or *oriC*, and it contains only one DnaA box. Instead, *oriCIIvc* contains many binding sites for RctB, a protein encoded by a gene located adjacent to *oriCIIvc* (9). Even though the predicted 658-aa RctB protein lacks similarity to any characterized initiator and does not have any known motifs, several lines of evidence suggest that this protein may act as the initiator of chrII replication. First, this protein binds to several sites within *oriCIIvc* (9). Second, replication of an *oriCIIvc*-based minichromosome requires RctB (9), and the copy number of such a minichromosome increases as the levels of RctB are raised (11). Finally, overproduction of RctB in *V. cholerae* promotes overinitiation of chrII and not chrI (10), suggesting that RctB specifically triggers initiation at *oriCIvc*.

Here, we purified RctB and characterized its activities *in vitro*. We found that RctB has origin-specific unwinding activity and can melt *oriCIIvc* but not *oriCIvc.* Even though RctB lacks any ATP-binding motifs, this protein bound and hydrolyzed ATP. Surprisingly, ATP inhibited the *oriCIIvc* binding activity of RctB, suggesting that the ATP-bound form of RctB cannot initiate replication of chrII. Supporting this idea, we identified an RctB mutant that does not bind ATP and found that expression of this "ATP blind" RctB mutant in *V. cholerae* leads to significant overinitiation of chrII and marked inhibition of *V. cholerae* growth.

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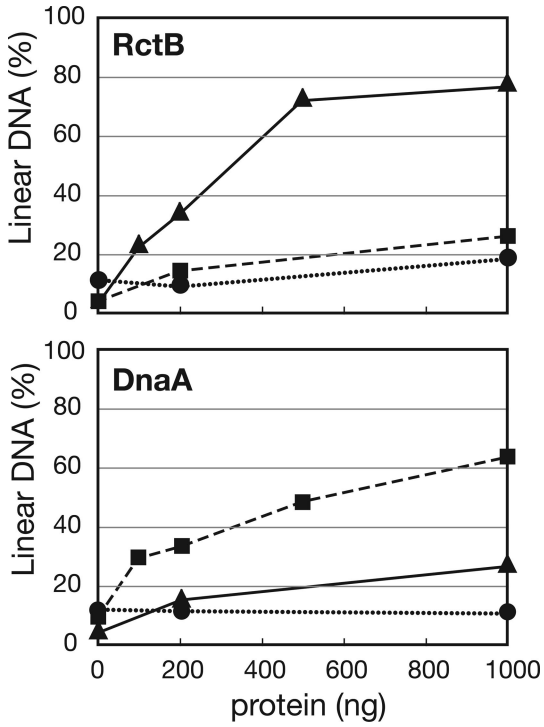

Fig. 1. *OriCIvc* and *oriCIIvc* unwinding activity of RctB and DnaA. Various concentrations of RctB (*Upper*) or DnaA (*Lower*) were added to reactions that contained 150 fmol of either pBR322 (●), pOriI (■), or pOriII (▲). P1 nuclease was subsequently added. Densitometric scanning of ethidium bromide-stained gels was used to determine the proportion of linearized plasmid molecules. The data presented are representative of at least three experiments.

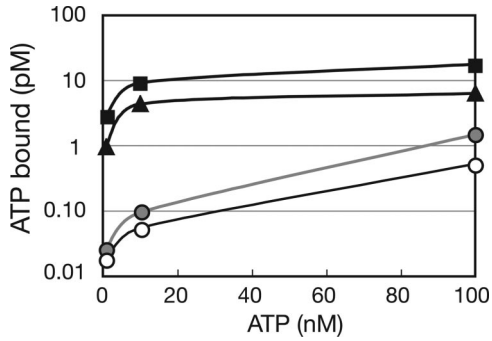

Fig. 2. ATP binding by RctB and DnaA. A total of 80 nM of DnaA (■), RctB (▲), CpxR (gray circles), a protein that is not known to bind ATP, or no protein (○) were incubated with different concentrations of P^{32}-αATP, and the bound ATP was detected by using a filter binding assay. A representative experiment of three experiments is shown.

Results

RctB and DnaA Promote the Unwinding of *oriCIIvc* and *oriCIvc*, Respectively. Previous genetic and cytologic studies revealed that RctB and DnaA are required for the replication of *oriCIIvc* and *oriCIvc*, respectively (9, 10). To gain understanding of the biochemical activities of these proteins, we used a P1 nuclease-based assay to test whether purified RctB and/or DnaA can unwind *oriCIvc* and/or *oriCIIvc in vitro*. In this assay, *V. cholerae* RctB or DnaA was incubated with circular plasmid DNA substrates that harbored either *oriCIvc* (pOriI), *oriCIIvc* (pOriII), or no chromosome origin (pBR, a negative control). If the protein promotes the unwinding of an origin sequence, the DNA becomes susceptible to cleavage by the single-strand specific P1 endonuclease. The resulting linearized plasmid is then easily detected by agarose gel electrophoresis. We observed that RctB unwound the *oriCIIvc*-containing but not the *oriCIvc*-containing substrate in a concentration-dependent manner (Fig. 1), demonstrating that RctB can indeed function as the initiator of *oriCIIvc*-based replication. In contrast, DnaA unwound the *oriCIvc*-containing substrate but had no activity on the *oriCIIvc*-containing substrate (Fig. 1), indicating that DnaA can function as the initiator of *oriCIvc*-based replication. DnaA + RctB did not exhibit greater *oriCIIvc*-unwinding activity than RctB alone (data not shown), suggesting that although DnaA is required for the *in vivo* replication of an *oriCIIvc*-based replicon (9), this protein does not contribute to melting *oriCIIvc* to initiate replication of chrII. Together these observations support the idea that the two *V. cholerae* chromosomes rely on distinct initiators for their replication: DnaA is the initiator of chrI replication and RctB is the initiator of chrII replication.

RctB and DnaA both Bind and Hydrolyze ATP. The predicted amino acid sequence of *V. cholerae* DnaA contains Walker A and B motifs and thus, like *E. coli* DnaA, *V. cholerae* DnaA is expected to bind and hydrolyze ATP. In contrast, RctB lacks motifs suggestive of nucleotide binding or hydrolysis. We used a filter-binding assay to test whether *V. cholerae* DnaA and RctB bind ATP. Both initiator proteins bound ATPα-P^{32} with similar K_d (DnaA, 43 nM; RctB, 39 nM) (Fig. 2). The affinity of DnaA and RctB for ATP is comparable to that reported for DnaA purified from *E. coli* (4) or *Mycobacterium tuberculosis* (12). We also used the same assay to test whether either of the two *V. cholerae* initiator proteins bound UTP, CTP, or GTP but neither RctB nor DnaA had detectable affinity for these triphosphonucleotides (data not shown). However, we found that ADP could compete with ATP for binding to RctB and DnaA [supporting information (SI) Fig. S1], suggesting that both proteins can bind ADP in addition to ATP (Fig. S1).

To test whether RctB and DnaA can hydrolyze ATP, we used an assay in which the conversion of ATP to ADP + Pi is linked to the oxidation of NADH to NAD+ (detected as a decrease in absorbance at 340 nm) (13). Both RctB and DnaA proved capable of hydrolyzing ATP (Fig. 3*A*). The calculated k_{cat} values of ATP hydrolysis for DnaA and RctB were similar ($k_{cat\ RctB}$, 0.41 min^{-1}; $k_{cat\ DnaA}$, 0.67 min^{-1}) and in the same range as has been reported for DnaA derived from other bacteria (12).

***oriCIIvc* DNA Inhibits RctB from Binding ATP.** Supercoiled DNA has been reported to stimulate the ATPase activity of *E. coli* DnaA in a sequence-independent fashion (4). We tested whether supercoiled plasmids influenced ATP hydrolysis by *V. cholerae* DnaA or RctB. Neither pOriI, pOriII, or pBR appreciably altered DnaA's hydrolysis of ATP (Fig. 3*B* and data not shown). However, the ATPase activity of RctB was inhibited by DNA in a sequence-specific fashion: pOriII almost completely abolished ATP hydrolysis by RctB, whereas pOriI and pBR did not alter RctB's hydrolysis of ATP (Fig. 3*B* and data not shown). These observations suggest that the *oriCIIvc*-bound form of RctB is unable to hydrolyze ATP. The *oriCIIvc*-mediated block in the ATPase activity of RctB appears to occur at the level of RctB binding to ATP. The presence of pOriII decreased RctB binding of ATP to near background levels, whereas pBR had little effect on RctB binding of ATP (Fig. 3*C*). In aggregate, these observations suggest that RctB binding to *oriCIIvc* renders the initiator unable to interact with ATP.

ATP Inhibits RctB Binding to *oriCIIvc*. Because *oriCIIvc* DNA inhibited RctB binding of ATP, we explored whether ATP inhibited RctB binding to *oriCIIvc*. In these experiments, RctB was preincubated with ATP or ADP and then electrophoretic mobility-shift assays (EMSAs) were done to test RctB binding to a

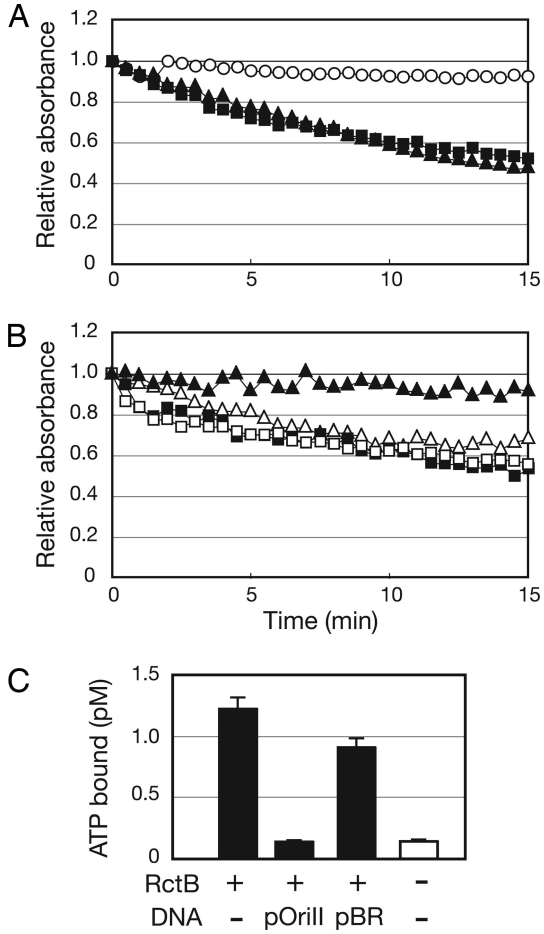

Fig. 3. Hydrolysis of ATP by RctB and DnaA. (*A*) Spectrophotometric assay of generation of ADP after incubation of 10 μM DnaA (■), 10 μM RctB (▲), or no protein (○) with ATP. (*B*) Influence of pOriCII DNA (triangles) or pOriCI DNA (squares) on ATP hydrolysis by DnaA (open symbols) or RctB (closed symbols). (*C*) Inhibition of RctB binding of ATP by pOriCII DNA. A total of 80 nM of RctB was incubated with 1 nM of P^{32}-αATP in the presence or absence of 180 ng of pBR or pOriCII DNA. A control experiment without RctB and DNA shows the background level of P^{32}-αATP binding to the filter. The results shown in *A* and *B* are representative of at least three experiments, and the data in *C* represent the average and standard deviation derived from three experiments.

DNA probe derived from the *oriCIIvc* region (Fig. 4*A*). Premixing RctB with ATP abolished RctB binding to *oriCIIvc* DNA, suggesting that ATP negatively regulates RctB's ability to function as an initiator. In contrast, premixing RctB with ADP did not appear to alter RctB binding to *oriCIIvc* (Fig. 4*B*). Together, these two observations suggest the possibility that hydrolysis of ATP bound to RctB could relieve ATP-mediated inhibition of RctB binding to *oriCIIvc*, thereby enabling RctB to initiate replication at *oriCIIvc*.

An RctB Mutant That Does Not Bind ATP Promotes Overinitiation at *oriCIIvc*. In a recent screen for high copy number variants of an *oriCIIvc*-based plasmid, we identified an RctB mutant that mediates high-copy *oriCIIvc*-based replication. Ordinarily, a minichromosome containing *oriCIIvc*, *rctB*, and a gene conferring resistance to kanamycin replicates with a very low copy number in *E. coli* (Fig. S2 and data not shown). In contrast, pYB286, which harbors a single nucleotide mutation in *rctB* codon 269, leading to a substitution of serine for arginine (R269S), replicates at a much higher copy number (Fig. S2). Quantitative PCR analyses revealed that the copy number of

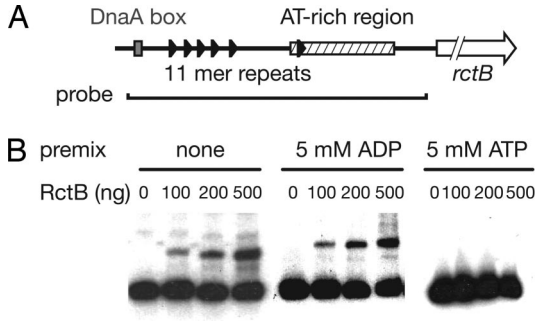

Fig. 4. Influence of ATP on RctB binding to *oriCIIvc*. (*A*) Schematic representation of the *oriCIIvc* region, AT-rich region (hatched area), DnaA box (gray rectangle), 11-mer repeats (black triangles), and *rctB* (arrow). The line below the map represents the probe used for the EMSA experiments. (*B*) Autoradiographs of EMSA experiments. The indicated molar quantities of RctB were preincubated for 10 min with either ATP or ADP. Afterward, the radioactive probe was added to the reaction.

pYB286 is >100 times greater than that of plasmids lacking the R269S mutation (data not shown). To gain further understanding of the activity and regulation of RctB, we explored how the R269S RctB mutant mediates high copy *oriCIIvc*-based replication.

We first tested whether the enhanced copy number of pYB286 could be attributed to elevated *oriCIIvc* unwinding activity by this protein. As was found with wild-type (WT) RctB, the mutant R269S RctB proved capable of unwinding *oriCIIvc* (Fig. 5*A*) but did not promote formation of linear pBR (data not shown). Although the P1 nuclease-based assay is only semiquantitative, comparison of the *oriCIIvc* unwinding activity of R269S RctB and WT RctB did not suggest that the mutant protein had elevated *oriCIIvc* opening activity (Fig. 5*A*). Thus it seems unlikely that the elevated copy number of pYB286 can be explained by enhanced *oriCIIvc* unwinding by R269S RctB.

Alternatively, the elevated copy number of pYB286 could result from altered regulation of R269S RctB activity. We tested whether the *oriCIIvc* DNA binding activity of R269S RctB was inhibited by ATP. In the absence of ATP, the binding of *oriCIIvc* DNA by R269S and WT RctB were comparable; however, unlike WT RctB, the binding of R269S RctB to *oriCIIvc* was not inhibited by ATP (Fig. 5*B*). Thus, R269S RctB appears to be insensitive to the inhibitory effect of ATP that was observed with WT RctB.

The lack of ATP inhibition of R269S RctB binding to *oriCIIvc* is likely attributable to a severe reduction in the binding of ATP to this protein. ATP binding to R269S RctB was markedly reduced compared with the binding of ATP to WT RctB (Fig. 5*C*). ATP binding to R269S RctB was only marginally above the background levels observed when no protein was used (Fig. 5*C*). These observations suggest that Arg-269 is a crucial residue for RctB to bind ATP. Together, the findings that R269S RctB has approximately WT *oriCIIvc* unwinding activity but greatly reduced affinity for ATP suggests that the absence of ATP inhibition of RctB binding to *oriCIIvc* accounts for the high levels of R269S RctB-mediated *oriCIIvc*-based replication in *E. coli*.

Overinitiation of Chromosome II Replication Blocks *V. cholerae* Growth. We introduced plasmids containing isopropyl β-D-thiogalactopyranoside (IPTG)-inducible forms of RctB or R269S RctB into *V. cholerae* strain N16961 to explore the effects of these proteins on *V. cholerae* morphology, chromosome replication, and growth. As previously observed, overproduction of WT RctB did not significantly alter cell morphology (10). In contrast, ≈20% of cells overproducing R269S RctB exhibited

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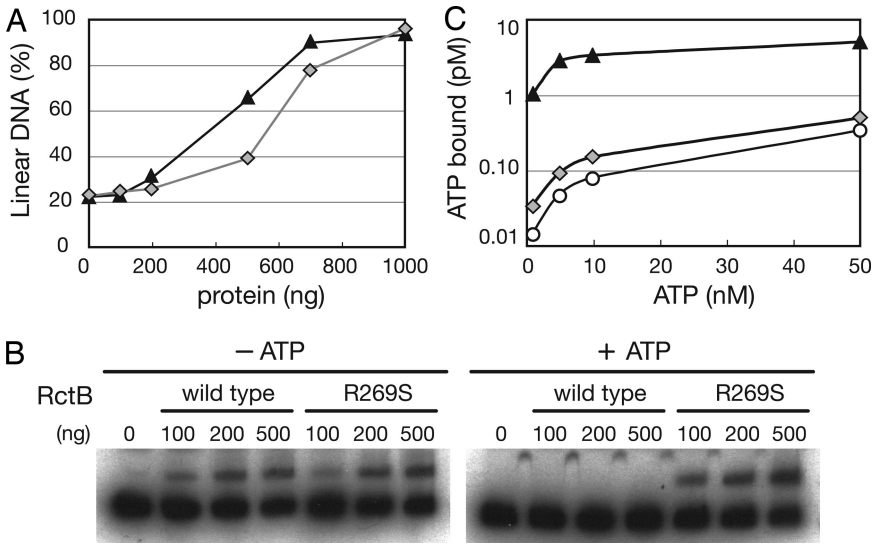

Fig. 5. *oriCIIvc* unwinding and binding of ATP and *oriCIIvc* by R269S RctB. (*A*) P1 nuclease cleavage assay on *oriCIIvc* DNA by WT RctB (triangles) and RctB R269S (gray diamonds). (*B*) EMSA of RctB and R269S RctB binding to the *oriCIIvc* probe in the presence or absence of 5 mM of ATP. ATP was mixed with RctB for 10 min before the addition of the *oriCIIvc* probe. (*C*) Binding of ATP by 80 nM RctB (triangles) or 80 nM RctB R269S (gray diamonds) using the filter binding assay as in Fig. 2; ○ represent ATP binding to the filter observed without the addition of protein.

filamentation (Fig. 6*A*), suggesting that R269S RctB blocks *V. cholerae* cell division. We used the fluorescent repressor-operator system (14) to evaluate the effects of overexpression of R269S RctB on the number of chrI and chrII origin regions in *V. cholerae*. As reported (10), overproduction of WT RctB led to a modest increase in the number *oriCIIvc* foci detected in most cells (Fig. 6*Ab*). In contrast, overproduction of R269S RctB led to a larger increase in the number of *oriCIIvc* foci (Fig. 6*Ac*). Nearly 11% of cells overexpressing R269S had six or more *oriCIIvc* foci, whereas <1% of cells overproducing RctB had this number of foci. Most of the cells with very large numbers (6–20 total) of *oriCIIvc* foci were filamentous (Fig. 6*Ac*). The number of foci representing the terminus of chrII (*terIIvc*) did not show a corresponding increase in cells overexpressing R269S; <4% of cells had ≥6 *terIIvc* foci (Fig. S3). Together, these observations are consistent with the idea that expression of R269S RctB leads to overinitiation of chrII replication *in vivo*. The effects of R269S RctB were chromosome-specific and no increase in the number of *oriCIvc* foci was detected in cells expressing R269S RctB (Fig. S3). The different effects of overexpression of WT vs. R269S RctB cannot be attributed to greater expression of the mutant protein, as Western blotting revealed that WT RctB was more abundant in these assays (data not shown)

Overproduction of R269S RctB profoundly inhibited *V. cholerae* growth. When overnight cultures of *V. cholerae* strains harboring IPTG-inducible forms of RctB or R269S RctB grown in the absence of inducer were plated in the absence or presence of IPTG (Fig. 6*B Upper* and *Lower*, respectively), ≈100-fold fewer colonies arose when R269S RctB was overproduced (Fig. 6*B*, compare rows c and f); in marked contrast, overexpression of WT RctB did not inhibit *V. cholerae* growth in this assay (Fig. 6*B*, compare rows b and e). Furthermore, when these overnight cultures were diluted in fresh media ± IPTG, there was no detectable growth of the cells overproducing R269S RctB for the 8-h culture period (Fig. 6*C*, filled blue diamonds). Together with the observations described above, these findings suggest that overinitiation of chrII replication mediated by R269S RctB is extremely toxic to cells, at least in part because it blocks *V. cholerae* cell division.

Discussion

There is scant knowledge of the mechanisms that govern chromosome replication in bacteria that have genomes consisting of more than one chromosome. Our findings strongly support the hypothesis that the two *V. cholerae* chromosomes have distinct initiators that function in an origin-specific manner: DnaA initiates replication of chrI and RctB initiates replication of chrII. Because these proteins are conserved in all sequenced species of vibrio and photobacteria, it is likely that DnaA and RctB function as chrI and chrII initiators, respectively, in all members of the Vibrionaceae and Photobacteriacea. In this work, we did not focus on the regulation of *V. cholerae* DnaA, but given the similarity of the *V. cholerae* and *E. coli* DnaA amino acid sequences (79% identity and 83% similarity) it is possible that at least some of the mechanisms that are known to govern DnaA activity in *E. coli* also control DnaA activity in *V. cholerae.* Unlike DnaA, RctB is conserved only in vibrio and photobacteria species, and it lacks any functional motifs. Our observation that RctB, like DnaA, can bind and hydrolyze ATP, suggested that ATP might regulate RctB activity in a similar manner as described for DnaA. However, we unexpectedly found that ATP inhibited RctB binding to *oriCIIvc*, suggesting that ATP-RctB does not function as the initiator of chrII replication. In marked contrast, only the ATP bound form of DnaA is able to initiate replication in *E. coli* (4).

To our knowledge, the negative regulation of an initiator protein by ATP is without precedent among bacterial initiator proteins. Furthermore, like DnaA, the characterized eukaryotic and Archaea initiator proteins are AAA+ ATPases that are only active in their ATP-bound forms and inactivated by ATP hydrolysis (1). RctB, although an ATPase, may use ATP hydrolysis in the opposite manner as other initiators, as a positive switch enabling its melting of the origin of *V. cholerae* chrII. Overall, our findings are consistent with a model in which ATP binding to RctB renders it unable to bind to *oriCIIvc*. The reason ATP-RctB does not bind *oriCIIvc* is not known. However, it is unlikely that ATP and *oriCIIvc* compete for the same binding site on RctB, because a truncated RctB fragment binds ATP but not *oriCIIvc* (data not shown). Instead, it is possible that ATP acts as an allosteric inhibitor of RctB. It is tempting to speculate that a cell-cycle-dependent factor regulates hydrolysis of ATP by RctB, thereby restricting the availability of active (non-ATP-bound) RctB and preventing excessive initiation of chrII repli-

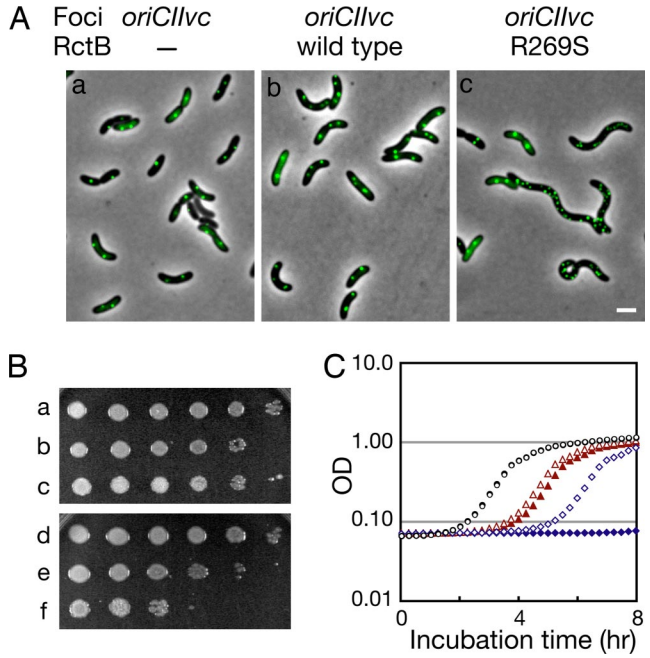

Fig. 6. *In vivo* characterization of *V. cholerae* overexpressing R269S RctB. (*A*) Detection by fluorescent microscopy of *oriCIIvc*, using TetR-YFP in an N16961 derivative that contains a *tet* operator array near *oriCIIvc*. Representative fields after 1 h of induction of TetR-YFP and RctB are shown. (Scale bar: 2 μm.) (*B* and *C*) Growth of *V. cholerae* N1691 with or without overexpressed RctB or R269S RctB. In *B*, overnight cultures of N16961 harboring a control vector (a and d), a vector containing WT *rctB* (b and e), or a vector containing *R269S rctB* (c and f) were serially diluted and spotted onto LB + chloramphenicol (5 μg/ml) with (d–f) or without (a–c) 100 μM IPTG. In *C*, the overnight cultures were diluted 1,000-fold into fresh media ± IPTG, and growth was monitored by absorbance at 600 nm. Control vector, no IPTG, (open black circles); control vector, + IPTG (filled black circles); WT *rctB*, no IPTG, (open red triangles); WT *rctB* + IPTG, (filled red triangles), R269S *rctB*, no IPTG (open blue diamonds), R269S *rctB* + IPTG (filled blue diamonds).

cation. While bound to *oriCIIvc*, RctB cannot bind ATP and is not subject to its inhibitory effects.

Our findings suggest that there must be a complex interplay between RctB, ATP, and *oriCIIvc* and perhaps other factors such as DnaA as well. We found that ATP inhibited RctB binding to *oriCIIvc* and conversely that *oriCIIvc* inhibited RctB binding to ATP. Unexpectedly, we did not detect significant ATP inhibition of RctB unwinding of *oriCIIvc* DNA using the P1nuclease assay (data not shown). This observation may suggest that the P1 assay is a more sensitive assay of RctB binding to *oricIIvc* than the EMSA, presumably because it reveals transient RctB-*oriCIIvc* interactions. *In vivo*, it seems likely that the relative levels and subcellular distribution of RctB, ATP, and *oriCIIvc* as well as additional factors determine when chrII replication begins. Future studies must define the factors that promote the formation and dissolution of the RctB–*oriCIIvc* complex. We did not find a role for DnaA in *oriCIIvc* unwinding *in vitro*, but DnaA is required for replication of an *oriCIIvc*-based replicon in *E. coli* (9). It is possible that DnaA promotes the loading of the replicative helicase (DnaB) on to *oriCIIvc*, especially because *V. cholerae* does not encode a homologue of DnaC, the *E. coli* helicase loader.

Although ATP appears to control the initiator activities of RctB and DnaA in opposite fashions, the two proteins seem to have several similar features. Both proteins are weak ATPases that may use ATP hydrolysis to regulate their activity. As for DnaA, the ATPase activity of RctB is not required for its origin-unwinding activity. We observed that WT RctB can melt *oriCIIvc* in the absence of ATP; furthermore, R269S RctB is able to melt *oriCIIvc* even though it does not bind ATP. Like R269S RctB, *E. coli* DnaAcos is defective for ATP binding and causes lethal overinitiation at *oriC* (7, 15). Overinitiation mediated by R269S RctB is expected if ATP negatively regulates RctB's interaction with *oriCIIvc*, whereas the reason DnaAcos mediates overinitiation at *oriC* is unclear.

Two studies have suggested that the replication of the two *V. cholerae* chromosomes is coordinated (16, 17). How could coordinated chromosome replication be achieved if ATP positively regulates *oriCIvc* unwinding by DnaA and negatively regulates *oriCIIvc* unwinding by RctB? Because we do not understand the other factors that regulate the activities of the two initiators of *V. cholerae* chromosome replication, it is possible that the opposite effects of ATP on DnaA and RctB do not impact the coordinated replication of the two chromosomes. Another potential explanation for this conundrum may be the proposal by Rasmussen *et al.* (17) that chrI and chrII do not initiate replication synchronously; instead they proposed that the two chromosomes terminate their replication synchronously. If this is the case, replication of some chrI-encoded product could provide a cue that triggers the hydrolysis of ATP bound to RctB, allowing chrII to initiate replication at a time that will ensure that the two chromosomes terminate replication synchronously. It is also possible that DnaA-ADP, generated by initiation of chrI replication, preferentially promotes initiation at *oriCIIvc*.

Materials and Methods

Protein Purification. *V. cholerae dnaA*, *rctB*, and *rctB* R269S were subcloned into the pET28b His-Tag vector (Novagen). The DNA sequence of each construct was experimentally confirmed. After expression of these genes in *E. coli* strain BL21, the cells were lysed in a French pressure cell and the N-terminal His-tagged proteins were purified by using His-trap nickel columns (Amersham) and FPLC as described (18). The purity of each of these proteins was >90% as estimated by SDS-PAGE.

P1 Nuclease Cleavage Assay. P1 nuclease cleavage of open complexes was carried out essentially as described by Bramhill and Kornberg (19). Each reaction (50 μl) contained 40 mM Hepes-KOH (pH 7.6), 8 mM magnesium acetate, 30% glycerol, 320 μg/ml BSA, 5 mM ATP, 150 fmol of supercoiled plasmid DNA, and different concentrations of RctB, RctB R269S, or DnaA. After incubation at 37°C for 10 min, P1 nuclease (1.2 units in 3 ml of 30 μM sodium acetate, pH 5.5) was added and incubated at 37°C for 30 s. P1 nuclease activity was then stopped by addition of 40 μl of stop buffer (25 mM EDTA, 1% SDS). For quantification of the fraction of the DNA that was linearized by the P1 nuclease, a portion of the reaction was electrophoresed in 0.8% TAE (40 mM Tris-acetate, 1 mM EDTA) agarose gel and then stained with ethidium bromide. Quantity One software (Bio-Rad) was used to determine the proportion of the plasmid DNA that was linearized by P1 nuclease.

ATP (ADP) Binding Assay. Binding of RctB and DnaA to ATP and ADP was assessed with a nitrocellulose filter binding assay essentially as described by Sekimizu *et al.* (4). Some 80 nM of protein (RctB, DnaA, or CpxR) was incubated in 40 μl of buffer E [50 mM Tricine KOH (pH 8.25), 0.5 mM Mn acetate, 0.3 mM EDTA, 7 mM DTT, 20% glycerol, 0.007% Triton X-100] with different concentrations of P^{32}-αATP for 15 min on ice. The reaction mixture was filtered onto a nitrocellulose membrane (Millipore HA 0.45 μM) presoaked in buffer E. The filter was washed with 10 ml of cold buffer F [50 mM Tricine KOH (pH 8.25), 0.5 mM Mn acetate, 0.3 mM EDTA, 5 mM DTT, 17% glycerol, 10 mM ammonium sulfate, 0.005% Triton X-100] and dried, and then the amount of radioactivity retained on the filter was measured in a liquid-scintillation counter.

ATP Hydrolysis Assay. Hydrolysis of ATP was monitored by using a coupled spectrophotometric assay as described by Huang and Hackney (13). The reaction buffer contained 25 mM Tris acetate (pH 7.5), 13 mM magnesium acetate, 1.8 mM DTT, 5 mM phosphoenolpyruvate, 20 units/ml pyruvate kinase, 20 units/ml lactate dehydrogenase, 5 mM ATP, 100 μg/ml purified BSA, 10 μM of protein (RctB or DnaA), 250 ng supercoiled DNA, and 0.3 mM NADH. The 100-μl reaction was set up directly in the cuvette of a Beckman DU 530 spectrophotometer at room temperature. The reaction was initiated by addition of ATP and then monitored for 15 min.

MICROBIOLOGY

***oriCIIvc* DNA Binding Assay.** Probes for electrophoretic mobility-shift experiments were amplified by PCR using chromosomal DNA prepared from *V. cholerae* strain N16961 as a template; P^{32}-labeled PCR primers (5′- CACTCAGGTTGTGGATAAAC-3′, 5′-GATCCGTATCACACTTACCG-3′) were used to label the probe. The labeled probe was purified from an acyrlamide gel, and the amount of P^{32} incorporated into the probe was determined by liquid scintillation counting. Twenty-microliter reactions were performed by incubating 5,000 cpm of probe DNA with different concentrations of RctB in a reaction buffer containing 200 mM Tris·Hcl (pH 7.5), 10 mM EDTA, 800 mM NaCl, 12.5 μg/ml sonicated salmon sperm, and 0.1 mg/ml of BSA for 10 min at room temperature. In some experiments, RctB was preincubated in a reaction buffer containing 5 mM of ATP or ADP. The reactions were then electrophoresed in a 6% DNA retardation gel and visualized by autoradiography.

Isolation of RctB R269S. We constructed a linear DNA molecule that consisted only of *rctA-oriCIIvc-rctB* and a kanamycin resistance gene. After *in vitro* ligation, attempts to recover a circular form of this DNA molecule after its transformation into *E. coli* DH5α routinely failed, presumably because *rctA* is a negative regulator of RctB (20). On the rare occasions when colonies arose after this transformation, they usually contained deletions in *rctA* (as in pYB289) or mutations in *rctB*. One of the mutant *rctB* sequences (in pYB286) was predicted to have a serine rather than an arginine at position 269 (R269S) and proved capable of mediating high copy replication of an *oriCIIvc*-based plasmid. The WT and *R269S rctB* genes were amplified by PCR and cloned into the EcoRI site of pGZ119EH (21), yielding pYB285 and pYB291, respectively. After confirming the nucleotide sequences of these plasmids, they were used for the studies shown in Fig. 6.

Microscopy. To observe cell and nucleoid morphology in *V. cholerae* strains harboring plasmids expressing WT or mutant *rctB*, cells were grown in LB broth until $OD_{600} \approx 0.1$ and then RctB was overexpressed by adding 100 μM of IPTG. After 1 h, cells were fixed with fixation solution (2.5% formaldehyde, 30 mM $NaPO_4$, pH 7.5). Cell nucleoids were stained with 250 ng/ml of DAPI. Images were captured and processed as described (18). *oriCIIvc* and *oriCIvc* were visualized as described (10, 14, 22). The *V. cholerae* strain containing *tetOP* array at *terIIvc* was constructed as described (22).

Plasmids and Strains. Plasmids and strains used in this study are listed in Table S1.

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