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Initiator protein dimerization plays a key role in replication control of *Vibrio cholerae* chromosome 2

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ABSTRACT

RctB, the initiator of replication of *Vibrio cholerae* chromosome 2 (chr2), binds to the origin of replication to specific 12-mer sites both as a monomer and a dimer. Binding to 12-mers is essential for initiation. The monomers also bind to a second kind of site, 39-mers, which inhibits initiation. Mutations in *rctB* that reduce dimer binding increase monomer binding to 12-mers but decrease monomer binding to 39-mers. The mechanism of this paradoxical binding behavior has been unclear. Using deletion and alanine substitution mutants of RctB, we have now localized to a 71 amino acid region residues important for binding to the two kinds of DNA sites and for RctB dimerization. We find that the dimerization domain overlaps with both the DNA binding domains, explaining how changes in the dimerization domain can alter both kinds of DNA binding. Moreover, dimerization-defective mutants could be initiation-defective without apparent DNA binding defect. These results suggest that dimerization might be important for initiation beyond its role in controlling DNA binding. The finding that determinants of crucial initiator functions reside in a small region makes the region an attractive target for anti-*V. cholerae* drugs.

INTRODUCTION

Dimerization represents one of the simplest ways to regulate protein activity post-translationally and is a widely used regulatory mechanism in many DNA–protein interactions (1–4). In the field of DNA replication, the regulatory consequences of dimerization have been studied extensively in a class of plasmid initiators (5). These initiators dimerize efficiently but generally bind to the origin of replication as monomers. Although inactive in DNA binding, the dimers serve important replication inhibitory functions not only by limiting monomer availability but also by bridging two origin-bound monomers to form a tetrameric bridge. The bridging is considered to be the major mechanism by which the sister origins are inactivated. Control of the association state of the initiators is therefore critical for regulating replication of these plasmids. The association state depends upon total protein concentration, which is controlled at the level of transcription, and upon post-translational remodeling by molecular chaperones (6–8).

Vibrio cholerae is among about 10% of sequenced bacteria that have divided genomes and currently provides the best-studied example of a bacterium in which a multipartite genome is maintained (9). One of its two chromosomes (chr2) appears to have evolved from a plasmid of the kind discussed above. In these plasmids and in chr2, the hallmark of the replication origin is the presence of an array of tandem repeats of initiator binding sites. The chr2 array has six repeats of a 12-mer that bind the chr2-specific initiator, RctB. The control of chr2 replication is, however, more complex than that of its presumed plasmid progenitor. For example, in addition to RctB monomers, RctB dimers bind to the 12-mers (10). Another novel feature of chr2 is the presence of a second kind of RctB binding site, 39-mers, which are strong inhibitors of chr2 replication initiation (11). Only RctB monomers bind to 39-mers (10). RctB mutants selected for increased initiator activity can have any or all of the following phenotypes: reduced dimer binding to 12-mers *in vitro* (10), reduced dimerization *in vivo* and reduced 39-mer binding both *in vitro* and *in vivo* (10–12). These results lead to the proposal that dimerization and 39-mer binding are inhibitory to replication, while monomer binding to 12-mers is stimulatory to replication (10,12).

We have embarked on a systematic structure-function analysis of the initiator protein RctB, which is known to mediate all known regulatory activities of chrII replication through binding to 12- and 39-mers. Thereby we aim to acquire a deeper understanding of chrII replication control, including an explanation for the following paradoxical observation: the RctB mutants that show increased monomer binding to 12-mers apparently due to dimerization defect show decreased monomer binding to 39-mers (10). Using primarily a reverse genetic approach, we show that the three basic functions of the initiator, namely DNA binding to 12- and 39-mers and dimerization, reside within a small re-

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gion covering about 10% of the protein, and that the dimerization domain overlaps with both the DNA binding domains. The overlap with the 39-mer binding domain suggests that the domain could be masked in dimers, making only monomers able to bind to 39-mers. The overlap also provides a ready explanation for how RctB copy-up mutants could be simultaneously defective in dimerization and 39-mer binding while promoting monomer binding to 12-mers. The dimerization-defective mutants could also reduce initiation either through reduced 12-mer binding or through some as yet unidentified mechanism (but not 12-mer binding). Dimerization thus appears to play a wider role in controlling chr2 replication than it is generally known to play in other DNA–protein interactions.

MATERIALS AND METHODS

Strains and plasmids

Escherichia coli, V. cholerae and yeast strains, and plasmids used in this study are listed in Supplementary Table S1.

Alanine substitution and deletion mutants of RctB

The alanine codon GCA was used in all substitution mutants to eliminate codon bias. The substitutions were generated using the overlap-extension polymerase chain reaction (PCR) method (13) with Fusion polymerase (10). The primers for PCR are described in Supplementary Table S2. The PCR template was the genomic DNA from WT *V. cholera*, N16961 (CVC209; Supplementary Table S1). The PCR products were cloned in pJJ85 vector between NdeI and XhoI sites for assaying promoter repression *in vivo*. The clones were verified by DNA sequencing.

Protein purification

To purify wild-type (WT) and mutant RctB proteins, the corresponding genes were cloned into the pTXB1 vector (14), and the derivative plasmids were used to transform BL21(DE3) for protein over expression. The proteins were purified essentially as described (10). RctB WT and mutants were also tagged with maltose-binding-protein by cloning the initiator genes into the pMal-c2X vector (14). These clones were used to transform BL21 cells for protein expression. Protein purification steps were same as used before (10).

Western blotting

Cell lysates were centrifuged at 16 000 *g* for 30 min in a microfuge, and the supernatant was loaded onto a 4–20% sodium dodecyl sulphate-polyacrylamide gel electrophoresis gel. Following electrophoresis, the proteins were transferred onto a nitrocellulose membrane and probed with RctB antibodies, as described (10).

EMSA

Plasmids (5 μg) were digested with restriction enzymes EcoRV and HpaI, and the resulting DNA ends were dephosphorylated with shrimp alkaline phosphatase (SAP) (Promega). The desired fragments were gel purified, radiolabeled with 30 units of polynucleotidyl kinase (15) and 50 μCi of adenosine 5′-[γ^{32}P] triphosphate (Perkin-Elmer), and purified by passing the mixture through G-50 columns (Roche Diagnostics Corporation). Electrophoretic mobility shift assay (EMSA) was done, as described (15).

Plasmid copy-number measurement

E. coli (DH5α) cells containing a plasmid source for WT or mutant RctB were further transformed with an *ori2*-plasmid (pJJ115). The copy number was measured using pNEB193 as the recovery control, as described (16).

Size-exclusion chromatography

Size-exclusion chromatography was performed using a Superdex 200 10/300 GL column (GE Healthcare) equilibrated with RctB buffer, and connected to AKTA FPLC (UPC900, Amersham Pharmacia Biotech). The column void volume was determined using Blue Dextran 2000 (17D4772). Proteins standards were obtained from Biorad (47–1901). The molecular mass of WT and mutant RctB proteins was calculated from the plot of gel-phase distribution coefficient (*K*av) versus log molecular weight of the standards. $Kav = (Ve\text{-}Vo)/(Vc\text{-}Vo)$, where Ve = elution volume, Vo = column void volume and Vc = geometric column volume.

Sedimentation velocity

RctB fragment C208-138 was studied in 0.5M NaCl, 0.025M Tris–HCl (pH 8.0) and 0.001M ethylenediaminetetraacetic acid (EDTA) at a loading concentration of 24 μM, whereas samples of C137, C157 and C208 were studied in 0.3M NaCl, 0.025M Tris–HCl (pH 8.0), 0.0005M EDTA and 5% (v/v) glycerol at loading concentrations of 3.6, 15 and 5.8 μM, respectively. Samples were loaded into 12 mm 2-channel epon centerpiece cells (400 μl) and temperature equilibrated at zero rpm prior to analysis. Sedimentation velocity experiments were conducted at 50 000 rpm and 20°C on a Beckman Coulter ProteomeLab XL-I using both the absorbance (280 or 230 nm) and interference (655 nm) optical detection systems following standard protocols (17). Data were analyzed in SEDFIT 14.4b (18) in terms of a continuous *c(s)* distribution covering a range of 0–5 S with a resolution of 100 and a confidence level of 0.68. Excellent fits were obtained in all cases. Protein partial specific volumes were determined in SEDNTERP 1.09 (19), as was the solution density and viscosity of 0.5 M NaCl, 0.025 M Tris–HCl (pH 8.0) and 0.001 M EDTA. The density and viscosity of the buffer solutions containing 5% (v/v) glycerol were determined experimentally using an Anton Paar DMA 5000 density meter and Anton Paar AMVn rolling ball viscometer at 20.0°C.

RESULTS

The C-terminal region of RctB participates in DNA binding

RctB is a 75 kDa protein and comprises 658 amino acid (aa) residues. It binds to two kinds of site, 12- and 39-mers, in the

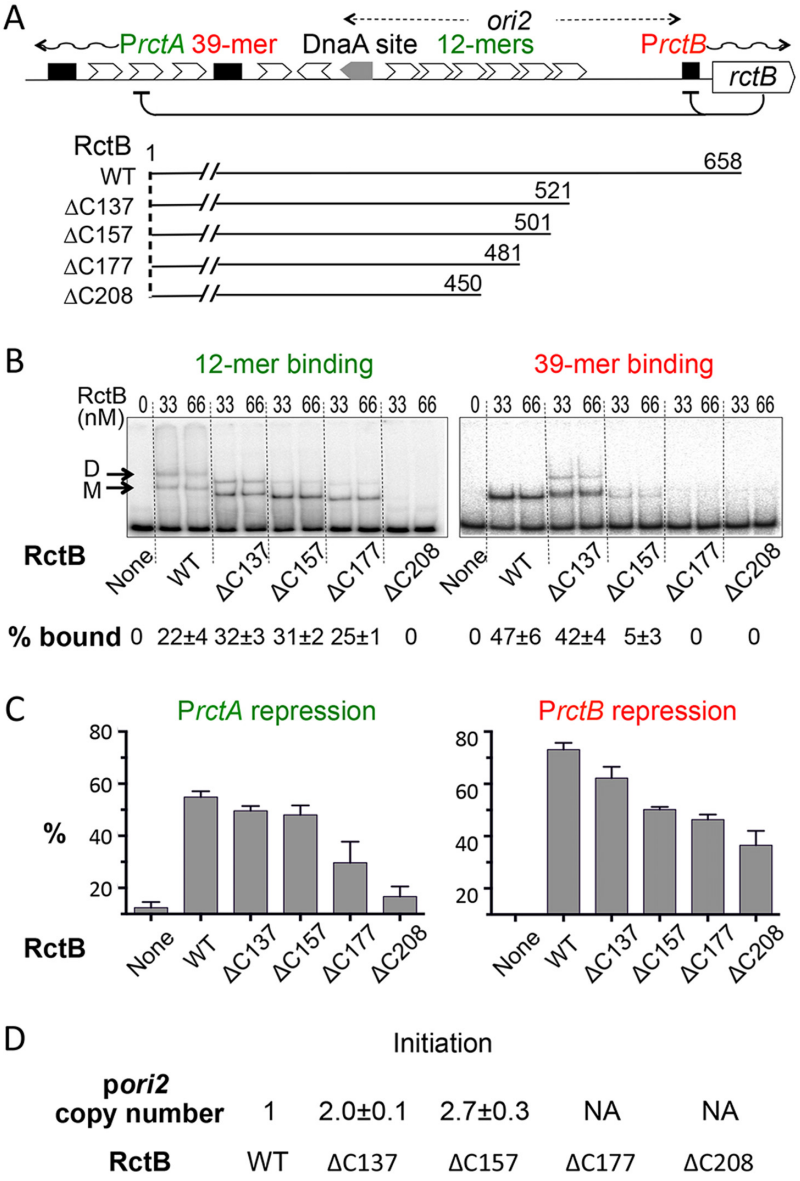


Figure 1. DNA binding and initiator functions of C-terminal deletion mutants of RctB. (**A**) Schematic map of the origin region of *Vibrio cholerae* chromosome 2 (chr2). The region has three functional units. One is *ori2*, the minimal region required for origin function. The hallmark of *ori2* is the presence of an array of six 12-mers (white arrow heads) where the chr2-specific initiator RctB binds. *ori2* also has a binding site for another initiator, DnaA (gray pentagon). The region to the left of *ori2* controls the origin activity. The region has extra 12-mers and another kind of RctB binding site, 39-mers (black rectangles), which are the primary inhibitors of chr2 replication. The region also has a promoter, P*rctA*, which RctB can repress by binding to overlapping 12-mers. The region to the right of *ori2* contains the *rctB* gene and its promoter, P*rctB*, which RctB can represses by binding to a 29-mer, a truncated 39-mer. Below the origin map is shown four RctB mutants deleted for C-terminal 137, 157, 177 and 208 amino acids (ΔC137, ΔC157, ΔC177 and ΔC208, respectively). (**B**) EMSA. End-labeled [^{32}P] DNA fragments (∼1 nM), carrying a single 12- or 39-mer with 55 bp flanks on both sides (obtained from plasmids pTVC195 or pTVC174 respectively), were reacted with WT and the four deletion mutants. The proteins were purified from pTVC16, pJJ21, pJJ03, pJJ20 and pJJ44, respectively. Lanes with no added RctB are marked 'None'. The binding mixture was analyzed in a 5% poly-acrylamide gel. RctB binds to 12-mers both as monomer (M) and dimer (D) (arrows), and their combined binding is used to calculate% bound DNA. RctB binds to 39-mers essentially as monomer.% bound = 100 × bound DNA/(bound + free) DNA. The mean binding from two lanes is shown. The mean ± one standard deviation values were determined from three independent gels. (**C**) Promoter repression *in vivo.* RctB binding to 12- and 39-mers *in vivo* was inferred from its ability to repress P*rctA* and P*rctB*, respectively (21). The promoters were fused to a promoter-less *lacZ* gene and one copy of the fusion was integrated into the *Escherichia coli* chromosome. RctB WT, ΔC137, ΔC157, ΔC177 and ΔC208 proteins were supplied from an arabinose-inducible promoter from pJJ69, pJJ71, pJJ70, pJJ82 and pJJ72, respectively. The relative levels of the proteins after induction were determined by western blotting (Supplementary Figure S1A). The empty vector (pJJ85) with the same inducible promoter was used as the negative control ('None').% repression = 100 × (β-galactosidase activity without arabinose − β-galactosidase activity with 0.02% arabinose)/β-galactosidase activity without arabinose. Three cultures inoculated with independent single colonies were assayed in each case, and the mean repression values with one standard deviation are shown. The relative levels of RctB upon induction with arabinose were determined by western blotting (Supplementary Figure S1A). (**D**) Initiator function *in* vivo. An *ori2* plasmid (p*ori2*; pJJ115) was used to transform cells (DH5α) containing one of the plasmid sources of RctB, as in (C). Copy numbers of p*ori2* were determined from the transformants using pNEB193 as recovery control (16). NA refers to situations where transformation with p*ori2* was unsuccessful.

origin of chr2 replication (Figure 1A) (10). *In vitro*, RctB monomers bind both sites, while dimers bind only 12-mers. Characterization of C-terminal deletion mutants of RctB provided the initial indication of the region important for DNA binding (10,12,20). The DNA binding of RctBΔC157 (deleted for the C-terminal 157 residues), mutant was characterized in the most detail. The binding to the 12-mer as monomer increased and as dimer decreased, indicating that the mutant is defective in dimerization. The mutant was also defective in 39-mer binding. To resolve this paradoxical monomer binding, decreasing in one case (39-mers) and increasing on the other (12-mers), we embarked on a study to define the DNA binding and dimerization domains of RctB.

To define the DNA binding domains, we constructed three mutants deleted for the C-terminal 137, 177 and 208 aa residues, called ΔC137, ΔC157 and ΔC208, respectively (Figure 1A), and monitored DNA binding *in vitro* by EMSA (Figure 1B). Similarly to the WT, ΔC137 was able to bind to a 12-mer both as monomer (M) and dimer (D), and to a 39-mer primarily as a single species, presumed monomeric. (There was an unexpected second retarded band of 39-mers, which could be due to presence of an altered folded form of the ΔC137 mutant, as argued in the 'Discussion' section.) The altered binding of ΔC137 compared to the WT indicates that the C-terminal 137 aa, although not essential for DNA binding, can influence it. The mutant ΔC177 showed a greater defect in 12-mer binding compared to the two shorter deletion mutants, ΔC137 and ΔC157, and binding to 39-mer was undetectable. The mutant with maximal deletion, ΔC208, showed no detectable binding to both the sites. The results indicate that some critical residues for DNA binding are located between deletion end points of the ΔC137 and ΔC208 mutants (in the interval 451–521 aa of RctB; Figure 1A). It also follows that the DNA binding domains are adjacent and that the 39-mer binding domain extends toward the C-terminus beyond the residues critical for 12-mer binding. These results are consistent with studies that suggested that the 39-mer binding activity resides at the C-terminus and it can be deleted without abrogating initiator function (12,20).

To test for DNA binding *in vivo*, we used a promoter repression assay (21) that utilizes the naturally occurring P*rctA* and P*rctB* promoters in the origin of chr2 (Figure 1A). P*rctA* has two overlapping 12-mers and P*rctB* has an overlapping 39-mer. Others and we showed earlier that repression of these promoters in *E. coli* by supplying RctB *in trans* provides a convenient assay for RctB binding to 12- and 39-mers (10,15,22,23). Also, to correlate DNA binding with initiator activity, the mutants were tested whether they can support chr2 origin-dependent plasmid (p*ori2*) replication in *E. coli*.

The ΔC137 mutant bound to both 12- and 39-mers *in vivo* nearly as well as the WT (Figure 1C), and increased replication efficiency of p*ori2* compared to the WT (Figure 1D). This could be because the mutant was relatively enriched in monomeric fraction as evidenced by the results of Figure 1B. The contribution of the C-terminal 137 aa is thus not critical for DNA binding and the region might have some inhibitory role in replication. In contrast, the ΔC177 mutant was significantly reduced in 12- and 39-mer binding compared to the WT, and failed to support p*ori2* replication. Western blot analysis using an RctB antibody indicated that the initiation defect is unlikely due to a reduction in initiator synthesis (Supplementary Figure S1A). We also considered the possibility that the replication defect is due to over-replication rather than no replication. However, over-replication was considered unlikely because we failed to recover transformants by providing extra titration sites, when the source of RctB was ΔC177. Reduced 12-mer binding appears sufficient to account for the initiation defect of the ΔC177 mutant, since decreased 39-mer binding is expected to promote initiation. This was also evident when DNA binding to an array of six 12-mers (6 × 12-mers) naturally present in the origin of chr2 was tested *in vitro* (Supplementary Figure S2A). The two mutants that were initiation defective, ΔC177 and ΔC208, were more defective in 6 × 12-mer binding than those that supported replication, ΔC137 and ΔC157. Because the ΔC157 mutant was proficient as an initiator and the ΔC177 mutant was not, the additional residues of the ΔC157 mutant appear to be critical for initiation, and most likely for optimal 12-mer binding. Overall, our *in vivo* results support the inference from *in vitro* work that the residues critical for both kinds of DNA binding are present in the 71 aa interval between the end points of ΔC137 and ΔC208 mutants. The fragment containing the 71 aa, spanning residues 451–521 (Figure 1A), is referred hereafter as C208-138.

Two presumed helical regions of RctB are important for DNA binding

Alanine substitution is often used in protein function analysis because it causes minimal perturbation to the overall folding of the protein backbone (13). To confirm the region critical for DNA binding, we analyzed the 71 aa region using alanine substitution. Secondary structure analysis of the region revealed two sequence stretches with high propensity to form α-helices (named H1 and H2; Figure 2A). The amino acids of those two sub-regions were changed to alanine and the resulting mutants were called mH1 and mH2. Both mutants were defective in binding *in vitro*, but the defects were of opposite specificities: mH1 was more defective in 12-mer binding and mH2 was more defective in 39-mer binding (Figure 2B). The double mutant mH(1 + 2) was defective in binding both the sites. These results are consistent with inferences made above that the 12- and 39-mer binding domains are adjacent to each other and that residues critical for 39-mer binding are more C-terminal to the residues critical for 12-mer binding (Figure 1).

The results of DNA binding of these mutants *in vivo* (Figure 2C) support the *in vitro* results, namely that mH1 is more defective in 12-mer binding, and mH2 is more defective in 39-mer binding. Testing of the initiator activity of the mutants revealed that only mH1 was defective, as would be expected since only the 12-mer binding is critical for initiation (Figure 2D). We confirmed that the mutants were produced in similar quantities, indicating that their functional differences are intrinsic to their changed amino acid composition (Supplementary Figure S1B).

To confirm that the defective initiator function of mH1 is correlated with its binding defect to 12-mers, we tested *in*

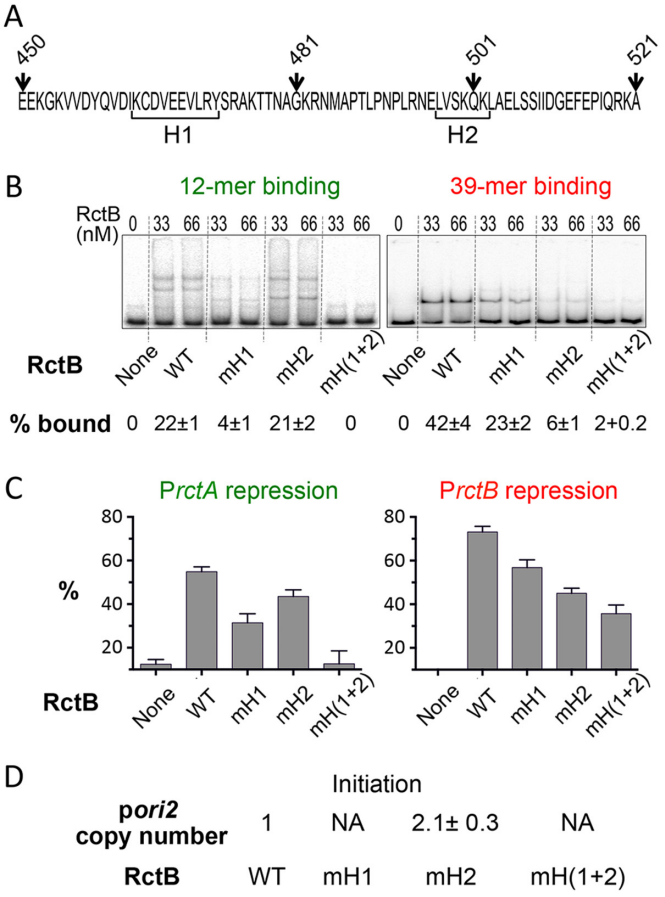


Figure 2. Importance of two putative helical regions of RctB in DNA binding and initiator functions. (**A**) Sequence of RctB residues 450–521. The residues include the region between the C-terminal ends of mutants ΔC208 and ΔC137, and an incidental E (the 450th residue). Secondary structure analysis of the interval predicted two regions of high helical propensity (brackets), referred to as H1 and H2. (**B**) EMSA. This was performed as in Figure 1B except that the mutant proteins were mH1, mH2 and mH(1 + 2), where H1 and H2 residues were all changed to alanine, and the changes were combined in mH(1 + 2). The proteins were purified from pJJ68, pJJ67 and pJJ86, respectively. (**C**) Promoter repression *in vivo*. The repression was measured as in Figure 1C except that RctB mutants were mH1, mH2 or mH(1 + 2). RctB WT and the three mutants were expressed from pJJ69, pJJ74, pJJ73 and pJJ87, respectively, and their relative amounts were determined by western blotting (Supplementary Figure S1B). (**D**) Initiator function *in* vivo. This was tested as in Figure 1D except that RctB WT and the mutants were from plasmids used in (C).

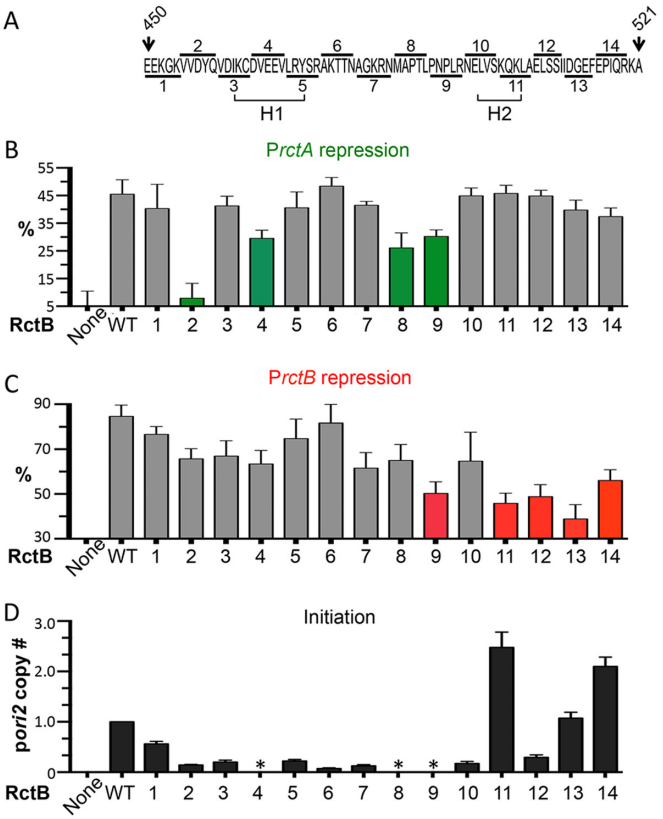


Figure 3. Alanine scanning mutagenesis of RctB residues 450–519. (**A**) Sequence of RctB residues 450–521, as in Figure 2A, showing residues changed to alanine five at a time (under- and over-lines) in mutants numbered 1–14. (**B** and **C**) Promoter repression *in vivo*. The repression was measured, as in Figure 1C, except that RctB proteins were alanine mutants #1–14. The mutant proteins were supplied from plasmids described in Supplementary Table S1, and their relative amounts were determined by western blotting (Supplementary Figure S1C). (**D**) Initiator function *in vivo*. This was measured as in Figure 1D, except that the RctB mutants were #1–14, as in (B) and (C).

vitro binding to the origin array of 6 × 12-mers (Supplementary Figure S2B). Mutant mH1 failed to saturate the 12-mers under the conditions WT and mH2 initiators could. The presumed helix H1 thus appears to be critical for 12-mer binding and replication of chr2. We later show that although H2 is not required for 12-mer binding, it might contribute to binding indirectly through RctB dimerization.

We also tested the initiator function of mH1 and mH2 in the native host, *V. cholerae* (Supplementary Figure S2C). It is known that excess of RctB can cause over-replication of chr2, which could be inhibitory to *V. cholerae* growth (20,24). When the mutants were over-produced, only mH2 could retard growth similarly to the WT RctB, consistent with the results in *E. coli* that showed higher initiator activity of mH2 over mH1. In conclusion, these results identify two presumed helical regions, one important for promoting initiation by 12-mer binding and another for inhibiting initiation by 39-mer binding.

Domains for 12- and 39-mer binding are contiguous

To test whether residues important for DNA binding extend beyond the presumed helices H1 and H2, we changed most of the 71 aa region to alanine, five residues at a time. Fourteen such mutants (#1–14) were constructed to cover the interval 450–519 (Figure 3A), and each mutant was characterized for DNA binding and initiator function. Mutants considered significantly defective in 12-mer binding (P*rctA* repression) contained changes within H1 (#4) and outside of H1 (#2, 8, 9) (Figure 3B). These four mutants were also most defective in binding to a single 12-mer *in vitro* (Supplementary Figure S3A). Mutants significantly defective in 39-mer binding contained changes within H2 (#11) and also outside of H2 (#9, 12–14) (Figure 3C). The same five mutants were also most defective in binding to a 39-mer *in vitro* (Supplementary Figure S3B). Since the mutant #9 was de-

fective in both 12- and 39-mer binding, the domains for 12- and 39-mer binding may be contiguous with an overlap of a few residues.

The binding data, however, were not always correlated with the initiator function of the mutants (Figure 3D). All four mutants defective in 12-mer binding (#2, 4, 8, 9) were initiation defective, as expected, but out of the four mutants (#11–14) considered specifically defective in 39-mer binding, only two showed the expected copy-up phenotype (#11, 14). Six other mutants (#3, 5–7, 10, 12) were initiation defective without an apparent defect in 12-mer binding. We also determined binding to the natural substrate, the 6 × 12-mers, *in vitro* (Supplementary Figure S3C). Again, DNA binding of many of the initiation defective mutants appeared normal (#1–3, 6–9). These results suggest that changes in the 71 aa region can compromise initiator function, not necessarily altering 12-mer binding. All mutants were expressed at levels comparable to that of the WT, indicating that the altered functions of the mutants were not because of their altered concentrations (Supplementary Figure S1C).

The 71 residues are sufficient for DNA binding

The results above indicated that the residues important in DNA binding are present in a 71 aa region but they did not say whether the region suffices for DNA binding. We initially tested whether a RctB fragment carrying the C-terminal 208 residues, 451–658 (Figure 4A), hereafter called C208, can bind DNA (Figure 4A). The C208 fragment failed to bind to a 12-mer (data not shown) or to a 39-mer (Figure 4B, lanes 15–17). To favor binding, we fused C208 to MBP (maltose binding protein), which can improve solubility/activity of its fused partner (25). Additionally, to improve 12-mer binding we used the origin array of 6 × 12-mers. Under these conditions, poor but significant binding could be seen both to 6 × 12-mers (lanes 8–10) and the 39-mer (lanes 18–20). Although the C208 fragment without the fusion was inactive in binding to a single 12-mer, it did bind weakly to 6 × 12-mer (lanes 5–7). The binding of MBP-C208 to both 12- and 39-mers was also significant *in vivo* (Figure 4C). These results indicate that the C-terminal 208 residues suffice for minimal DNA binding activity but optimal binding requires help from the N-terminal residues. Since fusion to MBP improved DNA binding, a role for the N-terminal residues could be in folding the region properly.

Even though the C208 fragment bound DNA poorly, our earlier observation that binding to the 12-mer improves when the C-terminal 137 aa are deleted (WT versus ΔC137; Figure 1B), prompted us to test binding with the C208-138 fragment. This 71 aa fragment could bind to both 12- and 39-mers *in vivo* and *in vitro* (Figure 4D and E). Although the binding was still poor, the results do indicate that minimal DNA binding activities reside within the 71 aa region.

The 71 aa residues are sufficient for dimerization

Both size exclusion chromatography and the capacity of RctB to bind as a dimer to 12-mers suggested that the initiator has dimerization activity. Since ΔC137 mutant was reduced in dimer binding relative to the WT and ΔC157

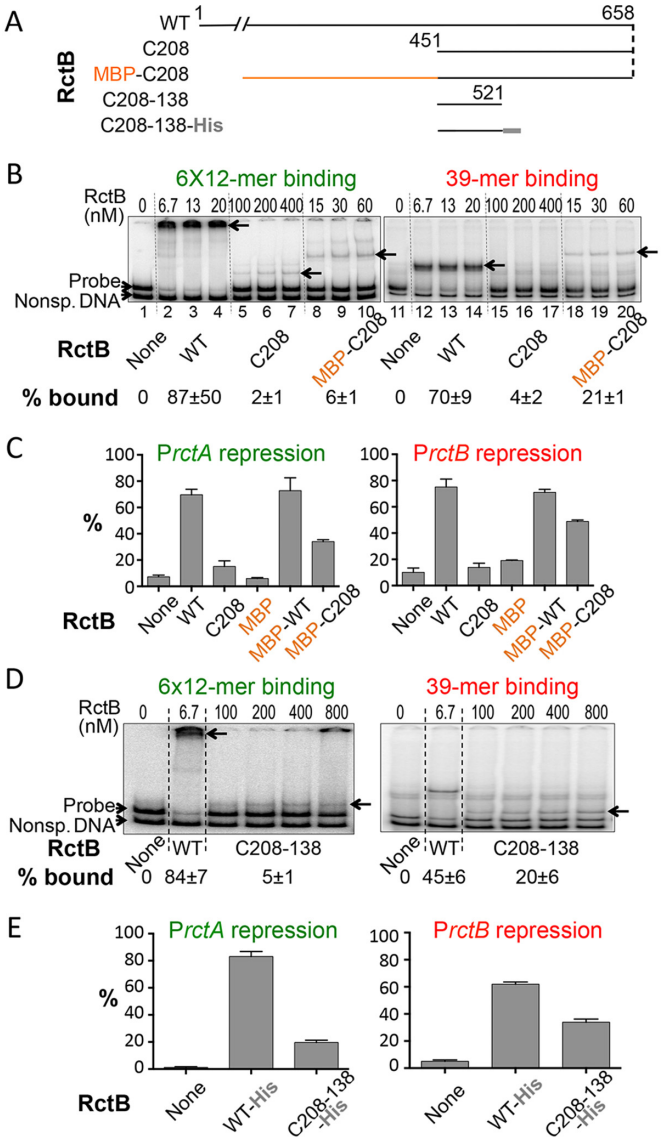


Figure 4. DNA binding of RctB C-terminal fragments. (**A**) Schematic map of RctB fragments tested. (**B**) EMSA. The C208 fragment either alone or fused in frame to the maltose binding protein (MBP), MBP-C208, contains RctB residues 451–658. The proteins were purified from plasmids pJJ25 and pJJ91, respectively. EMSA was performed as in Figure 1B. The DNA fragments (∼1 nM) were either the array of 6 × 12-mers of *ori2* (Figure 1A) or a single 39-mer, both with 100 bp of vector sequences at both flanks. The fragments were isolated from pTVC228 and pTVC174, respectively. (**C**) Promoter repression *in vivo*. This was measured as in Figure 1C except that RctB proteins were C208 and MBP-C208, as in (B), supplied from pJJ75 and pJJ91, respectively. WT RctB tagged with MBP, MBP-WT and the MBP moiety alone were used as positive and negative controls, and the proteins were supplied from pJJ56 and pMAL-c2X, respectively. MBP and its fused derivatives were induced with 10 μM IPTG and the rest including the vector control (pJJ85; 'None') were induced with 0.02% arabinose. (**D**) EMSA of C208-138 that contains 71 RctB residues in the interval 451–521. EMSA was performed as in (B) except that the RctB fragment was C208-138. (**D**) Promoter repression *in vivo*. This was measured as in Figure 1C except that RctB WT and C208-138 were tagged at their C-terminus with six histidines (−His), as shown in (A). They were supplied without induction from pJJ63 and pTVC14, respectively. The empty His-tagging vector (pET22b) was used as the negative control ('None').

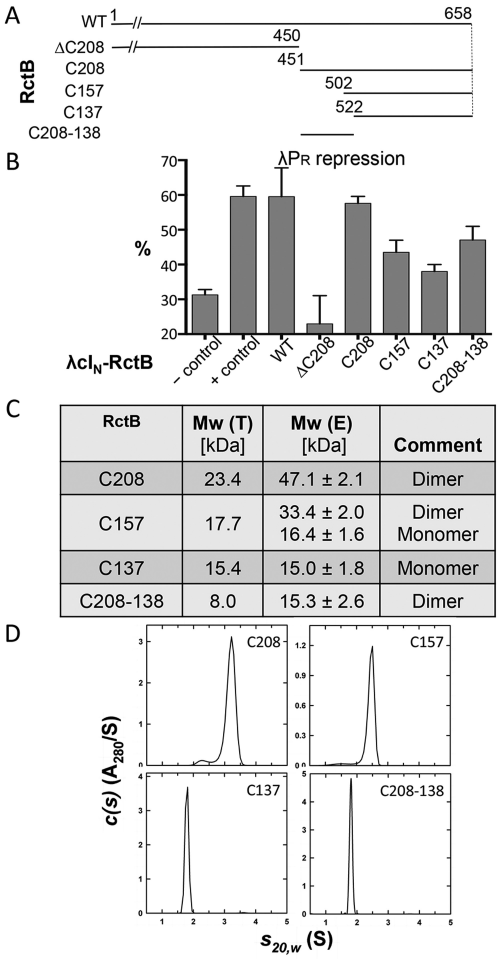
mutant bound to 12-mers almost exclusively as monomer, it seemed likely that the domain responsible for dimerization is at least partially included within the C-terminal 157 residues, 502–658 (Figure 1A).

We tested for dimerization activity of RctB *in vivo* using an assay based on the repression of the λP_R promoter by the λ immunity repressor (26). The repressor has two separate domains: an N-terminal DNA binding domain (λcI_N) and a C-terminal dimerization domain. The DNA binding activity requires dimerization, which can be provided by a dimerization domain from a heterologous source. We substituted the C-terminal domain of λ repressor with full length RctB, or the four deletion mutants of Figure 1A. The intact λ repressor (from pFG157) was used as positive control and λcI_N domain alone (from pOAC100) was used as negative control. All four deletion mutants were dimerization defective (Supplementary Figure S4).

To test whether a dimerization domain could be present within the C-terminal 208 residues, λcI_N was fused to the C-terminal fragments C208, C157, C137 and C208-138 (Figure 5A). λcI_N was also fused to a fragment of RctB containing the N terminal residues 1–450 (ΔC208) as a control. We found that both RctB WT and the C208 fragment were dimerization proficient, and as efficient as the λ repressor in repressing the λP_R promoter (Figure 5B). The ΔC208 fragment was as defective as the negative control, indicating that the RctB dimerization activity comes from its C-terminal region (Figure 5B and Supplementary Figure S4). The two smaller fragments, C157 and C137, appeared dimerization defective, particularly the C137 mutant, suggesting that the dimerization domain is likely to be within the 71 aa fragment C208-138. This was indeed the case (Figure 5B).

The dimerization activity from the C-terminal region of RctB was independently confirmed *in vivo* using the yeast two-hybrid system and crosslinking with dimethyl adipimidate dihydrochloride (DMA). For the two-hybrid analysis, the C208 fragment was fused to the LexA DNA-binding and GAL4 activation domains using the appropriate bait and prey vectors. When both the vectors carried the C208 fragment, expression of the reporter gene (leu2) could be detected, indicating that the fragment can self-associate (Supplementary Figure S5A). For the cross-linking assay, His-tagged versions of C208, C157, C137 and C208-138 fragments were used. The evidence for dimerization was robust for C208, weak for C157 and even weaker for C137 (Supplementary Figure S5B). The 71 aa fragment, C208-138, was also dimerization proficient although our RctB polyclonal antibody was inefficient in detecting this fragment.

The dimerization activity was further tested *in vitro* by purifying the above four His-tagged fragments, and subjecting them to gel filtration analysis (Figure 5C). Molar masses were determined from peak positions of the elution profiles using commercial protein standards (Supplementary Figure S6). The C208 fragment eluted as a single species and the peak of the distribution corresponded to a mass of 47.1 ± 2.1 kDa, as would be expected if the protein were a dimer (Figure 5C). The C157 fragment distribution was broader and elution peaks corresponded to both dimeric (33.4 ± 2.0 kDa) and monomeric (16.4 ± 1.6 kDa) forms of the fragment. The C137 fragment eluted in a single peak as would be expected if it were a monomer (15.0 ± 1.8 kDa). The 71



RctB	Mw (T) [kDa]	Mw (E) [kDa]	Comment
C208	23.4	47.1 ± 2.1	Dimer
C157	17.7	33.4 ± 2.0 16.4 ± 1.6	Dimer Monomer
C137	15.4	15.0 ± 1.8	Monomer
C208-138	8.0	15.3 ± 2.6	Dimer

Figure 5. Dimerization of RctB C-terminal fragments. (**A**) Schematic map of RctB fragments tested. (**B**) λP_R promoter repression by λ repressor-RctB fusions *in vivo*. In this assay, repression depends upon dimerization of the repressor protein. To assay RctB dimerization, the DNA binding domain of the λ repressor (λcI_N) was fused in frame to WT or N450 (carrying residues 1–450), C208, C157, C137 and C208-138 fragments of RctB. The fused proteins were supplied from plasmids pJJ112, pJJ113, pJJ93, pJJ94, pJJ95 and pJJ145, respectively. Additionally, the intact λ repressor and its DNA binding domain alone (λcI_N) were used as + and − controls, and supplied from pFG157 and pOAC100, respectively. The protein expression was induced with 10 μM IPTG. % repression = 100 × (β-galactosidase activity without IPTG − β-galactosidase activity with IPTG)/β-galactosidase activity without IPTG. Additional details are in Figure 1C and in (26). (**C**) Molar mass of RctB fragments C208, C157, C137 and C208-138 determined by size exclusion chromatography. This was performed using Superdex 200 10/300 GL column equilibrated with the RctB buffer (10). Void volume of the column was determined with Blue Dextran 2000. The column calibration and molar mass determination of the proteins was performed as described (10). MwT and MwE refer to molecular weights predicted from amino acid sequence, and from chromatography, respectively. (**D**) Sedimentation velocity analysis of RctB. Sedimentation *c(s)* profiles are shown for RctB fragments C208, C157, C137 and C208-137. The absorbance data in the case of C208 and C137 were collected at 230 nm, whereas for the other two, the data were collected at 280. Similar profiles were observed for the interference data. C208 showed a major species at 3.21S having a molar mass of 41.5 kDa, indicative of a dimer. This species may be in reversible exchange with the monomer observed at 2.29S. C157 showed a major species at 2.52S having an estimated molar mass of 33.4 kDa, indicative of a dimer that may be in reversible exchange with the traces of monomer observed at 1.73S. C137 showed a single species at 1.86S with a molar mass of 16.7 kDa, indicative of a monomer, and C208-138 showed a single species at 1.81S with a molar mass of 16.7 ± 1.1 kDa, indicative of a dimer.

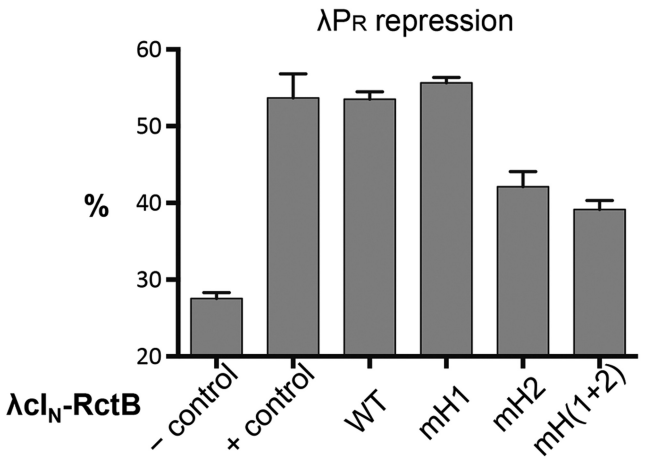


Figure 6. Importance of H1 and H2 regions in RctB dimerization as determined by λP_R promoter repression *in vivo*. Dimerization was assayed as in Figure 5B except that λcI_N was fused to RctB mutants mH1, mH2 or mH(1 + 2), and the fusion proteins were supplied from JJ129, pJJ128 and pJJ130, respectively. The control plasmids were same as in Figure 5B.

aa fragment, C208-138, also eluted in a single peak that corresponded to the mass of 15.3 ± 2.6 kDa, consistent with it being dimeric. These inferences were confirmed by sedimentation velocity experiments (Figure 5D).

Although the results were consistent overall, the C137 fragment showed some dimerization activity *in vivo* but not in solution studies *in vitro* (Figure 5, B versus CD). We note that in the *in vivo* study, dimerization was assayed when C137 was bound to DNA through λcI_N. DNA binding may have elaborated a separate weak dimerization activity that could be present within C137. Since ΔC137 bound efficiently to a single 12-mer but less so to the 6 × 12-mer array (Supplementary Figure S2A), some protein–protein interactions (as in cooperative binding) might be involved in efficient binding to the array and these interactions could be the basis for the weak dimerization activity seen in the λP_R promoter repression assay.

The dimerization and DNA binding domains overlap

We found that the mH2 mutant, which was defective in 39-mer binding (Figure 2), was also defective in dimerization when tested by the λP_R promoter repression assay (Figure 6). The defect was not because the mutants were unstable due to fusion to λcI_N (Supplementary Figure S1D). These results indicate that the changed residues in mH2 are important both for 39-mer binding and dimerization, as if the two domains overlap.

To define residues flanking H2 that could be important for dimerization, we tested dimerization activity of all 14 alanine-substitution mutants in the 71 aa region (Figure 3). The mutant clones were individually ligated in frame to the λcI_N fragment and the resultant fusions were assayed for their ability to repress the λP_R promoter. Seven mutants (#2, 3, 6, 7, 10, 12 and 13) were considered dimerization defective (Supplementary Figure S3D). The results indicate that residues that are important for dimerization reside in regions that are also important for binding to 12- and 39-mers (Figure 7A). Both kinds of DNA binding thus can be affected by dimerization.

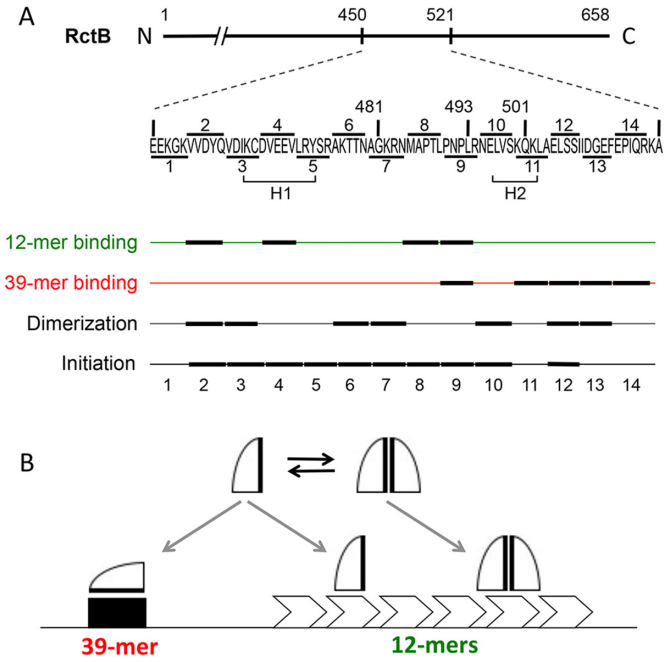


Figure 7. Summary of RctB regions important in DNA binding, dimerization and initiation. (**A**) The interval 450–521 of RctB is expanded to show the positions of changes in the mutants studied here. The C-terminal end points of deletion mutants are shown by vertical lines, substitutions in alanine mutants (#1–14) by under- and over-lines, and in H1 and H2 by brackets. The five residue patches of alanine substitutions that affected 12- and 39-mer binding, dimerization or initiation are shown by thick lines under the respective alanine chnages. The results show that the two DNA binding domains are next to each other with some overlap (#9), and both overlap with the dimerization domain. The residues important in dimerization interfere with 12-mer binding (#2) as well as with 39-mer binding (#12,13). The results help to explain (#1) how dimerization defect can cause 39-mer binding defect and (#3) how deletions up to 165 C-terminal residues are possible without impairing initiation function (vertical line at 493) (12). The deletion mutant does not cover any of the substituted regions found to be important for 12-mer binding, which is to be expected since 12-mer binding is essential for initiation. It covers residues important for 39-mer binding and dimerization, supporting the view that these two activities serve normally inhibitory roles in replication. The present results also show that although the deletion of the C-terminal 165 residues is tolerated, some of the alanine substitutions in the deleted region could cause initiation defect (#10,12). The dispensable C-terminal residues of RctB therefore can be exploited to influence essential functions of RctB in relation to arresting Vibrio *cholerae* growth. (**B**) Summary of origin binding activities of RctB. The cartoons show that RctB active in DNA binding equilibrates between two forms, monomer and dimer. The monomers have two faces, one for 12-mer binding and the other for 39-mer binding. Although residues important in dimerization map within both the DNA binding domains, only the 39-mer binding face is apparently occluded upon dimerization since both monomer and dimer bind to 12-mers and only monomer binds to 39-mer.

DISCUSSION

The replication initiator protein of chr2, RctB, binds to two kinds of DNA sites, 12-mers and 39-mers, to promote and inhibit replication, respectively. The protein also dimerizes. Here we show that important residues required for both kinds of DNA binding and dimerization are present in a small region spanning only 71 of the 658 residue-long initiator. The domains for 12- and 39-mer binding are next to

each other and may even overlap by a few residues. Some of residues important for both kinds of DNA binding were also important for dimerization. These results indicate the dimerization and DNA binding domains overlap, and one domain can control the activity of the other. The results help to explain some of the basic findings of chr2 replication control, as we discuss below. The interplay between DNA binding and dimerization could be a holdover from an earlier plasmid mode of maintenance, where the dimerization domain overlaps with one of the DNA binding domains (2,27,28). The interplay is a widely used regulatory mechanism in many DNA–protein interactions (1–4,29).

Multiple roles of initiator dimerization in the control of chr2 replication

Dimerization in iteron-based plasmids and chr2 serves several functions in addition to controlling monomer availability. Dimerization is a homeostatic mechanism analogous to transcriptional autorepression; they both dampen monomer level increase when protein or gene dosage increases, as occurs normally upon replication (30). The damped increase helps to prevent premature initiation when monomers are rate-limiting for initiation. In R6K plasmid and in chr2, the dimers also compete with monomers for binding to the origin. Because monomer binding is required for initiation, this helps to delay initiation (10,31). The dimers in plasmids also link two monomer-bound origins, forming a tetrameric initiator bridge (16,32). The bridging of origins is considered to be the major regulatory mechanism that prevents plasmid over-replication. Bridging also occurs between chr2 origins but it is not known whether dimers participate there (11).

Dimerization could also be playing additional roles in the control of chr2 replication. In chr2, the primary negative regulators are 39-mers, to which RctB binds as monomer. The overlap of the dimerization and 39-mer binding domains provide a simple structural explanation for how mutants could be simultaneously defective for dimerization and 39-mer binding. In an earlier study, several copy-up RctB mutants were found to be dimerization defective in a bacterial two-hybrid assay and, simultaneously, 39-mer binding defective using a *rctB* promoter repression assay (12). The correlation of these two activities can now be interpreted simply from the overlap of the domains for the two activities. Since the 39-mers bind monomers, the dimerization thus could be reducing the inhibitory role of the 39-mers indirectly by reducing monomer availability and directly by occluding the 39-mer DNA binding domain (Figure 7B). These can be considered positive regulatory roles of dimerization.

The overlap of 39-mer and dimerization domains can complicate interpretations of the basis of the copy-up phenotype. Mutants, such as ΔC157, were thought to be copy-up because of their reduced binding to the 39-mers, but it could now also be attributed to reduced dimerization, which would promote initiation-stimulatory monomer binding to 12-mers. These two activities are uncoupled in the alanine mutant #14 (Figure 7A), which is normal for dimerization but defective for 39-mer binding. Its copy-up phenotype thus can be attributed to reduced 39-mer binding unambiguously.

Mutants with the opposite phenotype, normal or somewhat reduced in 39-mer binding but defective in dimerization, were also found (e.g. alanine mutants #3, 6, 7; Figure 7A). These mutants are initiation defective even though they appear normal for initiation-promoting 12-mer binding and, if any, somewhat reduced in initiation-inhibitory 39-mer binding. Thus, dimerization defective mutants can be initiation defective without affecting DNA binding. Although the underlying basis for this defect is not known, it suggests another positive regulatory role of dimerization. It is conceivable that dimers participate in an apparent cooperative binding to the 12-mer array and in intramolecular bridging of 12-mers, and these could be playing an obligatory role in initiation.

Multiple modes of control on initiator dimerization

Initiation of chromosomal DNA replication occurs only once per origin per cell cycle and, in spite of this low frequency, occurs with great certainty. In all well-studied cases, the robustness of the process appears to arise from a combination of several positive and negative regulatory mechanisms (33,34). One of the common negative regulatory mechanisms is initiator inactivation. In bacteria, this can be achieved through covalent modification (35), cofactor (ATP) hydrolysis (36,37), proteolysis (38), interactions with inhibitor proteins (39,40) and inhibition by dimerization (27) or polymerization (41). Many of these mechanisms are also found in eukaryotic replication control, which additionally include mechanisms such as phosphorylation and nuclear export of initiation factors (42,43). In systems where monomers provide the initiator function, as is the case in a large family of bacterial plasmids with repeated initiator binding sites and in the plasmid-like chr2 of *V. cholerae* with repeated 12-mer binding sites, the simplest mechanism of initiator inactivation is dimerization of the protein (30,44).

In chr2, multiple mechanisms control initiator dimerization. The level of dimers is controlled transcriptionally by autorepression (22,23). The dimerization activity is also controlled post-translationally by molecular chaperones, which improve all DNA binding, including dimer binding to 12-mers (10). DNA can also be an allosteric effector of dimerization, since the 39-mers *in trans* increase the bridging of 12-mers by RctB (11). Many of these features of RctB are also true in the case of plasmid initiators (45,46). However, unlike in plasmids, where the chaperones dissociate the dimers, in chr2 they promote dimer binding to 12-mers, further signifying the importance of dimerization in chr2 replication control. In this context, it is to be noted that mutations far removed from what we claim here to be a dimerization domain can alter the dimerization activity (12). Thus, there could as yet unknown mechanisms for controlling dimerization.

Influence of 12- and 39-mer DNA binding domains on each other

Our results also indicate that changes in one of the DNA binding domains can change the activity of the other do-

main. In mH1 and mH2, although one of the binding activities was affected more than the other, the other activity was affected nonetheless (Figure 2B). This was also evident from the binding of the double mutant, which was more defective in binding to 12-mers than mH1 (Figure 2A and Supplementary Figure S2B). Similarly, in several cases alanine mutants altered in binding to one site were also altered in binding to the other site (#2, 4, 8; Figure 3). The ΔC137 mutant was simultaneously affected for both the binding activities, although it lacks an apparently non-essential region (Figure 1B). One explanation for the linked activities could be the proximity of the two domains, which might facilitate structural changes in one domain to affect the other.

The connectedness of the two domains was also suggested from the stimulation of RctB bridging of 12-mers in the presence of 39-mers *in trans* (11). Recently, a chr1 site was found to increase initiation-promoting 12-mer binding and decrease initiation-inhibiting 39-mer binding (47). By altering both the initiation-promoting and initiator inhibiting activities of RctB, the chr1 site might be more effective when there is a small shift in the monomer/dimer ratio, than if it only affected one of the activities.

Structural pliability of DNA binding domains

As discussed above, the activity of DNA binding domains are easily modulated by DNA binding (11,48) and molecular chaperones (10). The latter study also revealed that upon unfolding and refolding, RctB forms two differently migrating species of retarded 12-mers (Figure 5 (10)). This indicates that the 12-mer binding domain can refold in two forms. In the present study, ΔC137 mutant also showed two retarded species of 39-mers (Figure 1B). Since MBP-fusions improved binding of C208, it is possible that residues N-terminal to C208 help the DNA binding domains to fold properly. The widely present helix-turn-helix motif for DNA binding also requires help from external sequences for activity (49). For example, in the case of λ immunity repressor, although the two helices of the helix-turn-helix (α2 and α3) make most of the operator contacts, the N-terminal helix (α5) involved in dimerization is also important for DNA binding (29).

When RctB was deleted one residue at a time, up to 165 (494–658) C-terminal residues could be deleted without loss of initiator function (the terminal essential residue 493 is marked in Figure 7A) (12). The deletion end point mapped at the edge of the region considered important for 12-mer binding, hence initiation. The C-terminal 165 residues include the overlap region between the 39-mer binding and dimerization domains, and the viability of the deletion mutant suggests that neither 39-mer binding nor dimerization are required for 12-mer binding and initiation. In view of these clear results, the initiation defect of alanine mutants #10 and #12 is unexpected. Although the changes were outside the region required for 12-mer binding, the mutants were defective in binding to 6 × 12-mers *in vitro*, which could explain their initiation defect. It appears that although deletion of C-terminal 165 residues is tolerated, substitution therein can perturb the region required for 12-mer binding and, hence, initiation. The mechanistic basis of this apparently allosteric effect remains to be understood.

Putative functions of RctB regions flanking the small region implicated in DNA binding and dimerization

RctB is unusually large (75 kDa) for bacterial initiator proteins, which are found usually in the 30–45 kDa range. The large size suggests that RctB is a multifunctional protein and may possess functions not commonly found in initiators. Many of the known functions of RctB have no assigned domains yet. For example, in addition to 12- and 39-mers, RctB binds to a third kind of site that is present in chrI and enhances chr2 replication (47). RctB binds to the array of 6 × 12-mers apparently cooperatively and bridges two 12-mers *in trans*. Other functions, based on examples of analogous plasmid initiators, may include interactions with DnaA and DnaB that aid in helicase loading (45,46). Like DnaA, RctB may also have a separate domain for binding to single-strands to stabilize origin-melting for helicase loading (48). RctB function is also modified by interactions with chaperones and specific DNA sites, such as 39-mers, suggesting the presence of disordered regions (10–12). The disordered regions may allow interactions with multiple partners to form the nucleoprotein complex that initiates replication (50).

RctB as a drug target

RctB is unique to the *Vibrio* genus and hence a suitably specific target for therapy (51). Although much remains to be determined regarding RctB structure–function relationships, our findings considerably narrow the domain of the protein that appears most relevant to its critical function and to the control of that function. Accordingly, we expect that the information presented here will aid in the development of *Vibrio*-specific antibiotic drugs.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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