Interactions of replication initiator RctB with single- and double-stranded DNA in origin opening of *Vibrio cholerae* chromosome 2

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ABSTRACT

Studies of bacterial chromosomes and plasmids indicate that their replication initiator proteins bind to origins of replication at many double-stranded sites and also at AT-rich regions where single-stranded DNA is exposed during origin opening. Single-strand binding apparently promotes origin opening by stabilizing an open structure, but how the initiator participates in this process and the contributions of the several binding sites remain unclear. Here, we show that the initiator protein of *Vibrio cholerae* specific to chromosome 2 (Chr2) also has single-strand binding activity in the AT-rich region of its origin. Binding is strand specific, depends on repeats of the sequence 5′ATCA and is greatly stabilized in vitro by specific double-stranded sites of the origin. The stability derives from the formation of ternary complexes of the initiator with the single- and double-stranded sites. An IHF site lies between these two kinds of sites in the Chr2 origin and an IHF-induced looping out of the intervening DNA mediates their interaction. Simultaneous binding to two kinds of sites in the origin appears to be a common mechanism by which bacterial replication initiators stabilize an open origin.

INTRODUCTION

DNA replication initiates at particular sites on chromosomes called origins of replication (1). Initiation requires the strands of the origin to be opened (2). Origin opening has been most thoroughly studied in bacterial replicons, primarily those belonging to the chromosome, phages and plasmids of *Escherichia coli* (3,4), although viral replicons in transformed cells have also been extensively studied (5,6).

Currently, strand opening at eukaryotic origins of replication is the subject of particularly penetrating analysis (7,8). Replication origins in bacteria are generally composed of two regions (1,9): one of initiator binding sites and the other of AT-rich sequences at which the origin opens, referred to as a DUE (DNA unwinding element) (10,11,12,13). DUE opening is required for loading the replicative helicase; however, in the absence of loading, the opening remains stable (2,14). A mechanism for stabilization has been proposed for the *E. coli* replication initiator DnaA, which while remaining bound to its double-stranded (ds) sites simultaneously captures an open strand of DUE (12) and so prevents reannealing. Later crystallographic studies of *Aquifex aeolicus* DnaA bound to an oligonucleotide have provided direct evidence that the AAA+ domain of DnaA (domain III) has single-stranded (ss) DNA binding activity, whereas domain IV binds dsDNA (15,16,17,18). The cocrystal structure also revealed that the oligonucleotide is stretched upon DnaA binding. Stretching provides a mechanism by which DNA strands can be opened, since it is equivalent to unwinding DNA strands (18,19). A ‘continuous filamentation model’ was proposed in which a dsDNA-bound polymer of DnaA monomers extends into an open strand of DUE by switching DNA binding domains from IV to III (7,18). Support for this model has come from *in vitro* studies of both *A. aeolicus* DnaA (18,20) and *Bacillus subtilis* DnaA (21,22).

The above model does not address the role of those ds-DNA sites in the *E. coli* origin, oriC, that are not adjacent to the DUE. In *oriC*, most ds sites are arranged in two arrays, but there are also important sites outside of the arrays, such as the DUE-proximal R1 site (23). R1 is separated from the nearest array by an IHF binding site over which DnaA does not bind (23,24). Accordingly, the authors proposed an ‘ss-DUE recruitment model’, in which the stretch of DNA that separates R1 from the nearest array is looped out with the help of IHF, which localizes R1 to the nearest array. Looping out of R1 provides a mechanism by which DnaA binds to R1 and opens it (24). The current study demonstrates that the initiator protein of *V. cholerae* chromosome 2 also has single-strand binding activity in the AT-rich region of its origin. Binding is strand specific, depends on repeats of the sequence 5′ATCA and is greatly stabilized in vitro by specific double-stranded sites of the origin. The stability derives from the formation of ternary complexes of the initiator with the single- and double-stranded sites. An IHF site lies between these two kinds of sites in the Chr2 origin and an IHF-induced looping out of the intervening DNA mediates their interaction. Simultaneous binding to two kinds of sites in the origin appears to be a common mechanism by which bacterial replication initiators stabilize an open origin.
closure is mediated by contacts between DnaA bound to R1 and a site within the nearest array, R5, which allows the DUE to interact with the array more effectively (23,24,25). Further analysis indicates that DnaA monomers bound to the ds sites simultaneously contact the ssDUE to form a ternary complex (25).

The ability of initiator binding to ds sites in promoting strand opening in DUE has also been proposed for phage and plasmid origins (26,27,28). The replication initiators belonging to the iteron family plasmids, RK2 and F, that lack an AAA+ domain show in vitro sequence-specific ssDNA binding in the DUE that is independent of any dsDNA binding sites (29). However, when dsDNA binding sites are present, the initiators form a ternary complex, as in the ssDUE recruitment model.

Vibrio cholerae has two chromosomes of which chromosome 2 (Chr2) is the first example of a bacterial chromosome whose initiation of replication requires an initiator other than DnaA (30). It is named RctB. The Chr2 replicon, although believed to have originated from an iteron family plasmid, has many distinguishing features (30,31,32,33,34). For example, RctB is twice the size of plasmid initiators, and binds to two sites unique to Vibrionaceae genomes: 39-mers, which were found to inhibit replication (35), and ctsS, which was found to connect the timing of Chr2 replication to that of Chr1 (36,37,38). There is also a nuanced control of initiation by the segregation protein, ParB2 (39). While the control of Chr2 replication has been studied in some detail, the basic question of how the origin opens remains largely unclear (40,41).

A single array of six 12-mer sites in the Chr2 origin, ori2, binds RctB. They are separated from the DUE by an IHF site. Here, we have sought to characterize their roles in the opening of ori2. We report that there are six perfect and interspersed repeats of a tetrameric sequence in DUE of ori2. The repeats are required for ori2 function and for simultaneous binding of RctB to both ds sites and ssDUE. Our findings provide straightforward evidence in support of the IHF-mediated ssDUE recruitment model (24), and suggest that ss binding and formation of a ternary complex with ds sites are common features of bacterial initiators.

**MATERIALS AND METHODS**

**Strains, plasmids and primers**

*Escherichia coli* DH5α(λ pir) (= CVC123) was used for standard plasmid manipulations and propagation, and *E. coli* BR8706 for transformation experiments (42). The strains used to test for IHF requirement were BR2845 (IHF+) and BR4543 (himA::Tn10) (43), and MC4100 (IHF+) and MC4100(hip3) (44). *Vibrio cholerae* strain used was CVC2099 (= MCH1) (45). All enzymes, and vectors pNEB193 and pTXB1 were from New England Biolabs (Boston, MA, USA). Antibiotics and their concentrations were as follows: ampicillin 100 μg/ml, spectinomycin 40 μg/ml and chloramphenicol 25 μg/ml (for *E. coli*) and 3 μg/ml (for *V. cholerae*). Arabinose was used at 0.02% (for *E. coli*) and 0.2% (for *V. cholerae*). Plasmids, and gene blocks and primers used are listed in Supplementary Tables S1 and S2, respectively. All cloned regions were confirmed by DNA sequencing.

**Electroporation of V. cholerae**

For preparing electroporant competent cells, a single colony of CVC2099 (= MCH1)/pTVC11, where the plasmid carried the rctB gene under Pbad control, was inoculated to 40 ml of LB with 40 μg/ml spectinomycin and 0.2% arabinose, and the culture was grown to log phase at 37°C. The cells were then washed first with 20 ml and then 10 ml of chilled 2 mM CaCl2, and finally suspended in 200 μl of 15% glycerol. For electroporation, 750 ng of pori2 plasmids were added to the 40 μl of electrocompetent cells and pulsed in 0.2-cm cuvettes using Bio-Rad Gene Pulsar set at 2.5 keV, 25 μF and 200 Ω (Bio-Rad, Hercules, CA, USA). One milliliter of cold LB was added immediately to the cuvette and the cells transferred to a culture tube and were grown at 30°C for 2 h without shaking. The cells were pelleted and plated on suitable antibiotic plates with 0.2% arabinose. The colonies were counted after 20 h of incubation at 37°C.

**Plasmid copy number measurements**

The copy number of pori2 was measured in the presence of a second plasmid, pctlB (= pTVC11), that supplies RctB from an arabinose inducible promoter Pbad (35). BR8706, where the areE transporter gene is expressed constitutively, was first transformed with pTVC11. Next, the BR8706/pTVC11 cells were made competent in the presence of 0.2% arabinose to ensure RctB supply and then transformed with pori2. After overnight growth under selection, the colonies were collected by washing the plates and OD600 of the cell suspension was measured. Cells were diluted to OD600 of 0.005 and grown to OD600 of 0.3. A total of eight OD600 units of cells were mixed with 0.8 OD600 units of separately grown cells carrying pNEB193. DNA was isolated from the mixed culture using QIAprep Spin Miniprep Kit (Qiagen Sciences, Gaithersburg, MD, USA) and recovered in 50 μl. Two hundred fifty nanograms of each DNA was separated on 1% agarose gel, stained with ethidium bromide and illuminated with UV. Plasmid copy numbers were measured at least from three independent biological replicates. DNA of mutant and the corresponding wild-type (WT) plasmid was run always in the same gel in duplicates. Copy numbers were determined after two normalizations. First, the band intensities of the WT and mutant plasmids were normalized with respect to the intensity of the recovery plasmid (pNEB193) and then the normalized intensities of mutant plasmids were further normalized with respect to that of the WT plasmid. The final normalized values from different gels were combined to calculate the standard deviation (SD). The SD values of different mutants were then averaged and used as the SD of the WT.

**Proteins**

RctB was purified from BL21(DE3)/pTVC16 cells, where the plasmid carried the rctB gene in pTXB1 vector. The details of purification were as described (46). DnaK was purified from *E. coli* as described (47) and DnaJ was purchased from MyBioSource (San Diego, CA, USA).
RctB binding to ori2 by fluorescence polarization and EMSA

Interaction of RctB with single strands of the AT-rich region of ori2 was captured in solution by fluorescence polarization using various FAM-labeled oligonucleotides (FSC107, FSC090, FSC087, FSC112, FSC113, FSC089). These HPLC-purified oligos were purchased from Integrated DNA Technologies (Coralville, IA, USA). To study interaction with ds AT-rich region, equal amounts of FSC107 (top strand, TS) and FSC090 (bottom strand, BS) were mixed in an annealing buffer (10 mM Tris, pH 7.5, 50 mM NaCl, 1 mM EDTA). Annealing was carried in a thermocycler at 95°C for 3 min and gradual cooling to 25°C during a 45-min period.

For determining RctB binding to its ds sites, a 186-bp fragment carrying the 6×12-mer array was obtained by PCR amplification using pJJ114 as a template and SC009 or FSC009 (FAM-labeled) as a forward primer and JJ378 as a reverse primer. The DNA was methylated by incubating in 1× Dam buffer with 0.032 mM S-adenosyl methionine and 1 unit of Dam methylase at 37°C for 2 h in a reaction volume of 40 μl. The methylase was heat inactivated at 65°C for 20 min. Methylation status of the FAM-labeled DNA was checked using restriction enzymes DpnI, MboI and Sau3A that digest only methylated, only unmethylated, and both the DNAs, respectively.

For polarization measurements, increasing amounts of RctB were added to 1× EMSA buffer (20 mM Tris acetate, pH 7.5, 100 mM potassium glutamate, 10 mM magnesium acetate, 5% glycerol, 0.1 mM EDTA) containing a 1.5 nM FAM-labeled probe (3 nM in case of the annealed product), 200 nM poly(dI-dC), 1 mM dithiothreitol, 100 μM ATP, 200 ng DnaJ and 400 ng DnaK in a final volume of 100 μl. Four nanomolar unlabelled 6×12-mer fragment was added when desired. The reaction was incubated at room temperature for 15 min. The samples were then transferred to glass cuvettes for polarization measurements at 25°C using the Infinite M1000 Pro plate reader (Tecan, Maennedorf, Switzerland). Each experiment was repeated three times and mP values were plotted against RctB concentration. Absolute values were plotted in Figure 5 and normalized values (with respect to WT for mutated strands) in Figure 7. Values from different set of experiments were combined to calculate the SD.

The above samples were also analyzed by EMSA. After the polarization measurements, 15 μl of the samples were removed from the cuvette and loaded onto a 5% native polyacrylamide gel and electrophoresed at 12 V/cm in 0.5× TBE. The gel was scanned using Typhoon FLA 9500 (GE Healthcare, Marlborough, MA, USA). The image was analyzed using Fiji software (48).

IHF binding to ori2 by EMSA

The IHF site of ori2 was cloned in the vector pTVC61 that generated pTVC146. For EMSA, the probes were generated from these plasmids that carried ∼100 bp of DNA from both flanks of the cloning site essentially as described (35, 49). The probes were generated by PCR amplification using vector-specific primers TVC164 and TVC165. The product from pTVC61 was used as a nonspecific probe and the one from pTVC146 as a specific probe. The probes were identical except for the IHF site insert (ori2 925-89) in the latter. The PCR products were gel purified and total of 5 pmol ends of each probe were labeled in 50 μl with 8.5 pmol [γ-32P]ATP (50 μCi/reaction; PerkinElmer, Waltham, MA, USA) using 30 units of T4 polynucleotide kinase for 30 min at 37°C. The enzyme was inactivated for 15 min at 65°C and the fragments were purified using Sephadex G-50 column (0.8 ml bed volume; Roche, Indianapolis, IN, USA). The amount of labeled DNA was determined by TCA precipitation (Mallinckrodt Baker, Phillipsburg, NJ, USA) using 20 μg salmon sperm DNA (Invitrogen, Carlsbad, CA, USA) as a carrier. EMSA was performed using 2.5 nM labeled probes and purified IHF, as described (43). IHF protein was a gift from Steve Goodman (Nationwide Children Hospital, Columbus, OH, USA).

KmO4 footprinting

KmO4 footprinting assays were performed essentially as described (21). The plasmids used, pJJ114 (WT) or pSOC88 (tetramers 3–6 mutated), were isolated using QiaGen Plasmid Maxi Kit. The plasmid preparation was then treated with exonuclease V (RecBCD) for removal of genomic DNA contamination, and then further purified by gel extraction. KmO4 reaction mix was in 1× EMSA buffer containing 1 mM dithiothreitol, 100 μM ATP, 200 ng DnaJ and 400 ng DnaK and 840 ng of the desired plasmid in a final volume of 75 μl. RctB and IHF were added to final concentrations of 50 and 100 nM, respectively. The reactions mix was then incubated at room temperature for 30 min. KmO4 treatment was performed at 37°C for 2 min. KmO4 footprinting was performed at 50–100 nM concentration of RctB. After 5 min, the reaction was stopped by quenching with 6 μl of β-mercaptoethanol and the mix was immediately loaded on MicroSpin™ G-50 columns (GE Healthcare, Marlborough, MA, USA) equilibrated with water. The samples (∼100 μl each) were then treated with 0.05 μg proteinase K at 65°C for 15 min. The DNA was then purified by extraction with phenol:chloroform:isoamyl alcohol mixture and precipitation with ethanol. DNA was dissolved in 12 μl of warm water. Primer extensions were done in a 20 μl reaction mix consisting of 0.1 U/μl of Vent (exo-) DNA polymerase in manufacturer’s reaction buffer supplemented with 2 mM MgSO4, 200 μM each dNTP, 500 nM FAM-labeled oligonucleotide FSC009 or FSC008, and 450 ng of purified KMnO4-treated template DNA per reaction. The mix was initially denatured at 95°C for 2 min, followed by 35 cycles of extension (15 s at 98°C; 30 s at 55°C; 30 s at 72°C). Two microliters of the extension products were then mixed with HiDi and LIZ size standards and the mixtures were denatured at 95°C and loaded onto the 3730xl DNA analyzer (Eton Bioscience, San Diego, CA, USA). The data were analyzed using Peak Scanner™ Software v1.0 (Thermo Fisher, Waltham, MA, USA). Experiments were independently performed at least twice, and representative data are shown.

RESULTS

Minimal AT-rich region required for ori2 function

The features of the origin of Chr2, ori2, are as follows: a DnaA box, the 6×12-mer array, an IHF site and the AT-
rich region (Figure 1). A functional ori2 is contained within a stretch of 359 bp spanning Chr2 coordinates 775–1133 (Supplementary Figure S1), as in pTVC31 (49). Relative to the rest of the origin, the AT-rich region (965–1133) has remained largely uncharacterized. To determine the stretch of the AT-rich region minimally required for ori2 function, we have performed a deletion analysis of the region in E. coli, where it has been shown that ori2 supplied with a source of the initiator protein, RctB, can function (30). The use of E. coli avoids the incompatibility between the introduced ori2 plasmid (pori2) and the resident chromosomal ori2 that would occur in a normal Vibrio host. By the same token, use of the replicon-fusion strain MCH1, in which the entire Chr2 origin region is absent, is transformable by ppori2 (albeit inefficiently for reasons unknown) (45). Previous studies of truncated ori2 plasmids have found that the origin remains functional when the left end is deleted up to the beginning of the DnaA box and the right end is deleted up to the AT-rich region base pair 1094 (as in pTVC524) and base pair 1053 (as in pTVC33) (49,50). To test whether the AT-rich region can be shortened further, we made an isogenic set of pori2 plasmids with increasingly trimmed right ends and determined their capacity to transform E. coli and Vibrio strains.

We find that a pori2 plasmid deleted up to base pair 1027, as in pSOC41, functions in E. coli and V. cholerae, but pSOC44, a plasmid that has suffered a further deletion of 9 bp, lacks sufficient origin function to yield viable transformants in either host (Figure 1). We note that whereas transformants of E. coli and V. cholerae by plasmids with less severely trimmed origins grew equally, transformants of V. cholerae by pSOC41 required an extra overnight incubation to appear. This result indicates that the 8-bp stretch (AGATCAGT) present in pSOC28 but absent in pSOC41 helps but is not essential for ori2 function.

To determine whether the ori2 sequences were being maintained as autonomously replicating plasmids and not passively by integration into the chromosome, the transformants were tested for the presence of plasmids. Their presence was confirmed for pJJ114, pSOC28 and pSOC41, and their copy number measurements indicated that pSOC41 replicated as efficiently as pJJ114 in E. coli (Supplementary Figure S2). Note that the copy number of pSOC28 was higher than that of pJJ114. The higher copy number of pSOC28 was attributed to the absence of the 29-mer (essentially a truncated 39-mer), which acts as an inhibitor of replication (50). The lower copy number of pSOC41 compared to that of pSOC28 indicates that replication has been compromised in pSOC41. We also note that the ori2 fragment present in pSOC41 is identical to that present in pTVC34, which was found to be replication defective (49).

The reason for this discrepancy is most likely due to opposite orientations of the fragment with respect to the vector backbone in the two cases.

**Methylation at GATC sites of the AT-rich region is not essential for ori2 function**

Several features of ori2, the DnaA box, 6 × 12-mer array (iteron analogs) and IHF site, are also found in iteron family plasmids (13,51,52). However, ori2 has several additional features not found in plasmid origins, including a high density of GATC sites (30). Each 12-mer contains a GATC site whose methylation of the adenine residue by Dam methylase is essential for initiator binding, and hence functioning of ori2 (53).

There are also five GATC sites in the 169-bp AT-rich region of which the first two—the eighth and ninth—lie within the minimal functional origin (Figure 2A). When Gerding et al. mutated the first three GATC sites of the AT-rich region to GTAC, they found that the origin is inactivated, suggesting that methylation has an additional essential role in ori2 function besides its role in initiator binding to 12-mers (41). However, when we changed GATC to CATC, within either the eighth or the ninth GATC site of our WT plasmid, pJJ114, no significant effect on origin function was found. Origin function was determined by the ability of plasmids carrying the mutated origins to transform an E. coli host and replicate efficiently in the transformants (Figure 2B and C). The mutated plasmids were also able to transform the V. cholerae MCH1 host (Figure 2B). Our results indicate that the methylation of the GATC sites in the minimal AT-rich region is not essential for ori2 function. When two bases of the same GATC sites were mutated (from GATC to CTTC), the origin function was abrogated as would be expected from the earlier study (41). The difference in replication assay results of these different changes in GATC sites, each of which is expected to abolish methylation, is explained in the next section.

When the G to C substitutions in the eighth and ninth GATC sites of ori2 were combined, the plasmid carrying the mutant ori2 was still efficient in transformation and in replication, although with about half the efficiency of the WT in both the assays (Figure 2B and C). These results suggest that although not essential, methylation at GATC sites in the AT-rich region can augment ori2 function. This result is not surprising as DNA methylation is known to facilitate strand separation (53,54,55,56,57).

To confirm that G to C single changes in GATC sites indeed prevented their methylation, the methylation status of the mutated sites was analyzed by SMRT sequencing, which can detect base modification (Supplementary Figure S3). Base modification at adenines was indicated at all 12 GATC sites of ori2, except in the eighth and ninth positions if mutant. These results confirm that ori2, as in pSOC41, can function without methylation at GATC sites in the AT-rich region.

**The AT-rich region has tetramer repeats that are essential for ori2 function**

The AT-rich regions of bacterial origins often contain replicon-specific signature sequences in multiple copies (12). Even point mutations in these sequences can abrogate origin function (29,41,58,59,60,61). Here, we report the presence of such signature sequences in the AT-rich region of ori2: six direct repeats of 5’ ATCA, separated by an average of 7 bp (Figure 3A). These repeats are highly conserved across members of the Vibrionaceae family (Supplementary Figure S4).

To evaluate the importance of ATCA repeats individually, we substituted each ATCA with its complementary se-
Figure 1. Determination of minimal AT-rich region of ori2 required for replication initiation function. (A) A schematic map of the region important for replication of *V. cholerae* Chr2 (not to scale). The cis-acting region is called ori2, while the Chr2-specific initiator gene rctB (blue arrow) can be trans-acting. An IHF site (yellow box) divides ori2 into two parts. One contains a DnaA box (maroon), which is followed by an array of six 12-mers (green). The other part following the IHF site is the AT-rich region of the origin and includes a second kind of RctB binding site, a 29-mer (red square). GATC sites (red dots) are present throughout the origin. ori2 fragments that were tested for replication activity are shown below the schematic (black lines). The nucleotide coordinates of the ori2 fragment ends are also shown. The end positions are connected to the schematic by dotted lines. Plasmid names carrying these fragments (pori2) are also identified. (B) Table showing whether pori2 plasmids can transform hosts with an arabinose inducible source of RctB (pTVC11). The *E. coli* host was BR8706/pTVC11 and the *Vibrio* host was CVC2099/pTVC11. ‘+’ represents the presence of transformants and ‘−’ represents their absence. n.d. = not determined. The region spanning coordinates 775–1027 present in pSOC41 (boxed in red) is minimally required for ori2 function.

Figure 2. ori2 function is retained when two of the GATC sites in the AT-rich region are mutated to CATC. (A) A schematic map of ori2 showing the two GATC sites, designated eighth and ninth, that were mutated. Other details are as in Figure 1. (B) Transformation efficiency of pori2 WT (pJJ114) and its derivates carrying the GATC mutations in *E. coli* and *V. cholerae*. Mutated bases are shown in red. Mean ± SD values are from four biological replicate experiments. (C) Copy number of pori2 and its mutant derivatives from transformants in (B). Copy number of pJJ114 is shown with a black bar and that of mutants in white bars. The mutant copy numbers were normalized to that of WT cells. The mutations were G to C in the eighth, ninth or both the GATC sites as identified in the abscissa. The *P*-value was calculated by one-way ANOVA.

Sequence, TAGT. We mutated each tetramer individually and in combination, and measured copy number of plasmids with mutated origins. Mutating tetramer 1 or 2 alone rendered the origin nonfunctional, although mutations in the other four tetramers individually showed no significant effect (Figure 3B). Whereas tetramers 1 and 2 are necessary for origin function, they are not sufficient since the origin becomes nonfunctional when the other four tetramers (3–6) are mutated (Figure 3C). The origin with mutations in tetramers 4–6 was viable in *E. coli*, indicating that origin function minimally requires tetramers 1–3 (Figure 3C). However, the plasmid copy number is reduced, compared to WT, even when the first four tetramers are intact, indicating that optimal replication requires that either tetramer 5 or 6 be intact. In *V. cholerae*, requirements of the repeats for ori2 function seemed greater (Figure 3D). In sum, these results indicate that the tetramers are critical for ori2 function, although not all of them contribute equally: tetramers closer to the IHF site are more important for replication than those farther away from it.

To evaluate the contribution of individual nucleotides within an essential ATCA tetramer to its activity, we determined how transversion mutations in each nucleotide of the first tetramer affect ori2 function (Figure 4A). When
Figure 3. ATCA repeats in the AT-rich region are important for ori2 function. (A) Sequence of the ori2 region containing six ATCA (in bold) repeats, three of which overlap with GATC sites (red underlines). The repeats are labeled below their sequence with numbers 1–6 in bold. (B) Copy number of pori2 WT (black bar) and its six mutant derivatives (white bars) carrying ATCA to TAGT change in one of the repeats, as identified in the abscissa. Other details are same as in Figure 2. (C) Similar to (B), except that the mutant derivatives carried the ATCA to TAGT change in multiple repeats. (D) Transformation efficiency of some of the mutated plasmids in V. cholerae.

Figure 4. Importance of individual nucleotides of the first ATCA tetramer in ori2 function. (A) The sequence of the TS of ori2 region that contains the six ATCA repeats (in bold). The nucleotides of the first ATCA repeat and its two flanks G and T were mutated to their complements individually. The changed bases are in bold and color coded. Copy number of pori2 (pJJ114) and its mutated derivatives from transformants when obtained. Other details are same as in Figure 1. (B) Transformation efficiency of pJJ114 and its mutated derivatives in V. cholerae.

RctB binds to the T-rich BS of the AT-rich region and the binding increases in the presence of the 6 × 12-mer array

Inactivation of origin function by altering the tetramer sequences, without changing their overall AT content, suggests that they serve as protein binding sites and not merely as contributors to AT richness. DUE binding by two other initiators has been reported and the binding shown to be preferential for one of its strands (29,59), suggesting that RctB might also bind to tetramers in their ss state.

To detect ss binding, we separately labeled the TS and BS of the AT-rich region (coordinates 965–1053) with the fluorescent tag FAM (Figure 5A). Each strand was complexed with RctB and the mixture was analyzed by fluorescence polarization, which measures DNA–protein interactions in solution in real time. The results showed that RctB binds negligibly to the TS but significantly to the BS in a concentration-dependent manner (Figure 5B and C). In the presence of 6 × 12-mer (ds) fragments, binding of both the

the first base of the tetramer was changed from A to T, the plasmid carrying the mutated base failed to give any transformant. Transformants were obtained when the mutations were at the other three positions. Copy number measurements showed that plasmids with mutations at the second and third positions are significantly compromised in replication, while mutations at the fourth position lead to a less severe reduction in copy number. In contrast, mutations in the bases flanking the tetramer affected the copy number only marginally. In sum, these results indicate that the units of function are indeed tetramers and demonstrate that their bases contribute unequally to origin function, with the order of importance being A1 > C > T > A4. The order of importance of the bases was also supported when plasmids were used to transform V. cholerae (Figure 4B).

These results also explain how mutating GA of the eighth and ninth GATC sites could have inactivated the origin, as described earlier (41), since they also changed the most important base of the essential ATCA repeats 1 and 2 (Figure 2B).
strands of the AT-rich region improved, but more dramatically for the BS. These results indicate that RctB has ssDNA binding activity, which increases in the presence of the 6 × 12-mer array provided in trans.

When the above samples were analyzed by EMSA, the binding was apparent for the BS only and that also in the presence of the 6 × 12-mer array (Figure 5B and C, lower panels). Apparently, the majority of the binding seen by polarization was too weak to be captured by EMSA. Also, no binding to annealed TS and BS of the AT-rich region was apparent by either assay (Figure 5D). In sum, our results indicate that RctB binds specifically to the ss bottom (T-rich) strand of the AT-rich region, and that binding increases in the presence of the 6 × 12-mer array.

RctB, the 6 × 12-mer array and the BS of the AT-rich region form a ternary complex

The presence of the 6 × 12-mer array could increase RctB binding to the BS indirectly or directly. The affinity of RctB for the ssDNA could be indirectly increased by the 6 × 12-mer array acting to remodel the protein. This process would yield a binary complex of ssDNA and remodeled RctB. The affinity of RctB for the BS could also be directly increased by the formation of a ternary complex of 6 × 12-mer, RctB and the BS. RctB need not be in the remodeled form in the ternary complex. Multiple RctB molecules bound to the array could bind together to the BS, which would increase the overall affinity of binding (‘the linkage effect’) (24,25,62). A mixture of RctB, HEX-labeled BS and FAM-labeled 6 × 12-mer was subjected to EMSA and was analyzed for complex formation by sequentially scanning the gel for the HEX and FAM labels (Figure 6). When present alone, the HEX-labeled BS, like the FAM-labeled BS (Figure 5B), did not show any specific retarded complexes with increasing concentration of RctB in both HEX and FAM scans (Figure 6, lanes 1–5 and 16–20). The FAM-labeled 6 × 12-mer showed increased RctB binding with increasing concentration of RctB in the FAM scan only, as expected (Figure 6, lanes 21–25). When the two labeled fragments were present together, new retarded complexes were seen in the presence of RctB in scans for both FAM and HEX, and the locations of the complexes were identical in both the scanning channels (Figure 6, lanes 11–15 and 26–30). These results indicate that RctB can be bound to both the BS and the 6 × 12-mer array simultaneously, forming a ternary complex.

The role of tetramers in RctB interaction with the BS of the AT-rich region

To further explore the role of the tetramers in RctB binding, first three, first four and so on of the tetramers were mutated to their complementary sequences and RctB binding to the mutant BS was measured by florescence polarization and EMSA. As before, RctB was seen to bind to intact BS and the binding increased in the presence of 6 × 12-mer (Figures 5C and 7). None. In all cases, binding to the mutant BS probes decreased significantly in the absence of 6 × 12-mer (Figure 7, 1–3 to 1–6). In the presence of 6 × 12-mer, the increase in binding was unaffected when tetramers 1–3 of the BS were mutated. However, as more tetramers were mutated, the increase in binding mediated by the 6 × 12-mer was increasingly lost. These results indicate that not all the tetramers are required for RctB binding (Figure 3).

We note that in vitro, RctB binding to the BS occurred without tetramers 1 and 2, which were seen to be essential for ori2 function in vivo. An important difference could be that the repeats are provided as ssDNA in vitro, which presumably is not the case in vivo. The first two repeats could have a special role in initiating DNA opening. We return to this issue in the last subsection of the ‘Results’ section.

IHF is essential for ori2 function

Our results so far indicate that the affinity of RctB for the BS increases in the presence of 6 × 12-mer, apparently from formation of ternary complexes of 6 × 12-mer, RctB and the BS. In these experiments, the 6 × 12-mer array was supplied in trans to the BS. In ori2, however, the regions carrying the 6 × 12-mer and AT-rich region are in cis, and their interaction requires looping out of the intervening DNA. A putative site for the DNA-bending protein IHF in the intervening DNA would seem ideally positioned to facilitate such interactions. Its sequence, TTACAATATTTA, closely matches the consensus A/TATCAAnnnnTTG/A (63). If the interactions between the 6 × 12-mer and the AT-rich region seen in vitro are physiologically relevant, then IHF binding to its site in ori2 should be critical for ori2 function. Such a role of IHF was previously shown in the replication of plasmid pSC101 (64).

The role of IHF in ori2 function was tested by evaluating the ability of a pori2 plasmid (pJJ114) to transform an E. coli host mutated in himA, the gene for the α subunit of IHF (65). As controls, we chose from our lab collection an RSF1030-based plasmid (pST52) whose replication is independent of IHF and a pSC101-based plasmid (pML042), whose replication depends on IHF (43). While all three plasmids, pJJ114, pST52 and pML042, were able to transform the WT (himA+) host, only the IHF-independent pST52 plasmid was able to transform the himA mutant host (Figure 8A). When the mutant host was complemented with a plasmid supplying IHF, all three plasmids could transform the complemented host with equal efficiency. We note that the efficiency of transformation of the himA::tn10 host is an order of magnitude lower than that in the WT host. The poor transformability of the mutant host appears to be intrinsic, since the IHF-insensitive plasmid (pST52) also transformed the mutant host poorly, whether complemented or not. We also used another host mutated in gene for the β subunit of IHF, hip (MC4100 hip3) (44), and got similar results. Together, these results imply an essential role of IHF in ori2 function. We also confirmed specific binding of IHF to its site in ori2 by EMSA (Figure 8B).

We also sought evidence in vivo for interactions between the two flanks of the IHF site, one of which carries the 6 × 12-mer and the other the AT-rich region. A genetic test for interaction between distant sites mediated by DNA looping is typically done by inserting a half (5 bp) or full (10 bp) turn of a DNA helix between the sites. For a given location, if the insertion of half a turn, which alters the phasing between the flanking sites, abrogates the function but the insertion of a full turn, which maintains the phasing, does not, then
interactions between the sites are attributed to DNA looping. We found that insertion of 5 bp on one or other side of the IHF site abrogates origin function, whereas 10-bp insertions at the same locations do not (Figure 8C). These results indicate that the phasing between the AT-rich and 6 × 12-mer regions is important for origin function, presumably because it allows their direct interactions. The importance of phasing is also supported by insertion of 5 bp on both sides of the IHF site, which also abrogated the origin function. In this situation, the interacting surfaces are not expected to face each other (Figure 8D). This indicates that it is not sufficient to bring the interacting sites opposite each other; they need to face each other properly for ori2 function. Our results also show that phasing of the two flanks of IHF is equally important in E. coli and in V. cholerae (Figure 8C), which implies that IHF is essential in V. cholerae. However, ihf-deleted V. cholerae strains can be viable but the deletion apparently confers a growth disadvantage since insertions in ihfA and ihfB genes were recovered rarely in a high-density transposon mutagenesis study (66,67). The viability of IHF deleted strains can be due to the following: (i) Acquisition of suppressor mutation such as fusion...
of the two chromosomes or a mutation in \textit{rctB}, as has been reported in survivors of deletion of another gene that appears essential for \textit{ori2} function, \textit{crtS} (37,68). (ii) IHF may not be that critical for chromosomal \textit{ori2} function as measured by bacterial viability but be critical for \textit{ori2} function as measured by \textit{pori2} maintenance. In \textit{E. coli}, mutations in chromosomal \textit{oriC} (including some in the IHF binding region) that do not affect viability seriously interfere with \textit{oriC} plasmid maintenance bearing the same mutations (69,70). Further studies are required to clarify to what extent IHF is important for \textit{ori2} function.

\textbf{DISCUSSION}

\textit{ori2} opens at the two tetramers closest to the IHF site

From \textit{pori2} copy number measurements, it is evident that at least some of the tetramers of the AT-rich region are essential for origin function (Figure 3). To test whether the tetramers are involved in origin opening, we used KMnO$_4$ to probe for \textit{ori2} opening. KMnO$_4$ preferentially reacts to distorted or melted DNA and has been widely used to probe for such DNA at promoters and replication origins (71,72). KMnO$_4$ probing was done \textit{in vitro} using supercoiled plasmid DNA in the presence of purified RctB, IHF or both proteins. The plasmid used carried either the full-length origin (pJJ114) or the same origin in which tetramers 3–6 were mutated (pSOC88). KMnO$_4$ reactivity was maximal at the first and second tetramers of the BS when both RctB and IHF were present (Figure 9A, red arrows). The reactivity was near basal levels (i.e. reactivity in the absence of RctB and IHF) when the TS was probed (Figure 9B) or when repeats 3–6 were mutated (Supplementary Figure S5). These results are consistent with origin opening initiating at the tetramers closest to the IHF site, when both RctB and IHF are present.

Chr2 of \textit{V. cholerae} encodes its own initiator, RctB, which is the primary regulator of Chr2 replication. The regulation of Chr2 replication is as stringent as that of other well-studied chromosomes of bacteria, all of which are regulated by DnaA (31,32,73). Comparative studies of chromosomal replication control mediated by RctB and DnaA thus provide an opportunity to identify conserved features of control. Although the amino acid sequence of RctB is unrelated to that of DnaA, RctB, like DnaA, binds to ssDNA, preferentially to one of the strands of the AT-rich region (DUE) of the origin. The stability of ss binding increases greatly in the presence of RctB’s array of dsDNA binding sites (6 × 12-mers). The mechanism appears to involve direct coupling of the ds sites and ssDNA, which supports the ‘ssDUE recruitment’ model proposed for opening of the \textit{E. coli} origin (Figure 10) (24,25). The present results point to capture of primarily one of the open DNA strands, thus preventing its reannealing, as a well-conserved mechanism by which bacterial initiators stabilize origin opening. Initiator binding to origin ds sites thus plays a dual role by contributing to both initiation and stabilization of origin opening, as discussed below.
Figure 8. IHF is essential for ori2 function. (A) Transformation efficiency of pori2, the test plasmid, and derivatives of RSF1030 and pSC101, control plasmids that are known to replicate independently of and dependent on IHF, respectively. The hosts were either WT or mutated in one of the subunit genes of IHF (himA), or the same mutated host complemented with a plasmid carrying the two intact subunit genes of IHF. All the cells carried pSOC20 that supplied RctB from an arabinose inducible promoter. (B) EMSA showing the binding of IHF protein to the IHF site in ori2. The fragment carrying the ori2 IHF site (derived from pTVC146) had flanking sequences from the vector (pTVC61) in which the site was cloned. The fragment with the same flanking vector sequences without the IHF site was used as a control for nonspecific DNA binding. The fragments were end labeled with 32P. (C) DNA phase change experiment to determine interactions between the two flanks of the IHF site in ori2. Phasing was changed by inserting either a 5-bp sequence (smaller triangle) or a 10-bp sequence (larger triangle) at the same point of ori2 sequence (vertical dotted lines). Insertion points were in the left, right or both the flanks of the IHF site. The sequences shown cover from the last base of the 6 × 12-mer array (T) to the end of the first ATCA tetramer. The numbers 28 and 19 indicate the distance (in bp) between the 6 × 12-mer array and the IHF site, and that between the IHF site and ATCA repeats, respectively. (D) Diagram explaining how 5-bp insertions in both flanks, although maintaining the physical proximity of interacting sites (rectangles), can still abrogate their interactions because the sites may not be facing properly. The IHF protein is shown as a yellow round disc.

**Initiation of origin opening**

Common destabilizers of duplex DNA, such as DNA breathing, negative supercoiling and AT richness, cannot be sufficient for origin-specific opening. Additionally, there need to be some local factors. At origins from which replication proceeds in the theta mode, multiple ds sites appear to be the primary driver for significant opening at a DUE. The sites are believed to organize the origin into higher order structures that add sufficient stress to an origin already stressed by other destabilizers to initiate the opening (7,28,74,75,76,77). In ori2, the ds sites (12-mers) are arranged in phase and the participation of all six of them appears to be required for efficient binding, which is not achieved even with five sites (Supplementary Figure S6A and B). The result suggests that the array of six 12-mers is normally required to form the specialized structure that can promote origin function. The suggestion that the specialized structure normally requires the participation of all six 12-mers implies that its function be sensitive to their phasing. This prediction is supported by the finding that interrupting the array by insertion of 5 bp abolishes the origin function (41). The importance of phasing is also indicated when we searched for revertants of an inactive origin due to the 5-bp insertion (Supplementary Figure S6C). The inserted bases were deleted in the revertants that restored phasing of the 12-mers. Localization of the changes within the 6 × 12-mer array, and not elsewhere in the origin, indicates that the phasing of the array sites cannot be disturbed. We note that the revertants were selected under condition where the 39-mers (the primary negative regulators of ori2 function) were absent (35). This might have allowed the origin to function with fewer 12-mers. Another possibility is that RctB polymerizes beyond its specific binding sites, as has been seen in another study (78).

A second source of local stress can be from architectural proteins, such as IHF. By mediating DNA bending, these proteins can drastically reduce the energy input required to melt DNA (79,80). Another possible source of stress to DNA is its methylation at GATC sites. As noted in the ‘Results’ section, methylation of GATC sites not only reduces the melting temperature of DNA but can also destabilize DNA by bending it (53,54,55,56,57). We note that many sources of stress, although commonly at play in opening origins, can be dispensed with in vitro (22,24). Initiator binding to ds sites, however, is indispensable.
Figure 9. KMnO4 reactivity of the AT-rich region of ori2 WT (pJJ114) in vitro. Supercoiled pJJ114 either alone or after mixing with IHF and/or RctB proteins was allowed to react with KMnO4. (A) The reacted bases in the BS were monitored by primer extension using an FAM-labeled primer complementary to a region upstream of the 6 × 12-mer array. The positions of terminated extension products are shown in the electropherograms. The peaks were compared between the no-protein sample (red tracing) and the protein-added samples (blue tracing). The positions where signals in blue tracings were above the red tracing background are shown with upward arrows, both in the electropherogram and in the sequence of the AT-rich region of the TS. The sequence of the IHF site is highlighted in yellow, and the ATCA repeats in bold letters. The reactivity was most conspicuous in the region with the first two ATCA repeats. The background reactivity of the last ATCA repeat was high and adding protein did not change the reactivity significantly. The arrows are color coded to indicate reactive sites outside (yellow) or inside (red and green) of the ATCA repeat region. The inside reactive sites are in red when protein induced and in green when not. (B) Same as (A) except the reactivity to the TS was probed using an FAM-labeled primer complementary to the end of the AT-rich region. Protein-induced increase in KMnO4 reactivity to TS was more spread out than that was seen for the BS and included the IHF site. Additionally, there was decrease in KMnO4 reactivity (protein-mediated protection) in several places of the TS, especially over the fifth ATCA repeat [inverted (orange) arrows]. These results suggest the RctB and/or IHF modulate(s) accessibility of KMnO4 to both the strands, although ATCA-specific KMnO4 reactivity was seen more in the BS (A).

Stabilization of origin opening

That initiators of replication bind to ssDNA is now evident in all cases where ss binding has been studied. This includes DnaA of E. coli, B. subtilis, A. aeolicus and Thermotoga maritima (18,20,21,22,24,59), the initiators of plasmid RK2 and F (29) and RctB of V. cholerae (present study). The ss binding can now be considered an integral property of bacterial initiators. The origin of V. cholerae Chr1 (ori1) is extremely similar to E. coli oriC (30) and also expected to use the ssDUE recruitment strategy. Because Chr1 is not in competition with Chr2 for initiation, as they have different initiator proteins, there is no disincentive to use the same ssDUE recruitment strategy. ori1 and ori2 are also sufficiently different that the strategy might have evolved independently.

In the case of DnaA, ss binding of initiator proteins requires the initiators’ ds binding sites (18,21,22,59,81,82,83). The ds sites were proposed to promote ss binding in the following ways: A polymer of DnaA on an array of ds sites can serve as the nucleation center for loading new DnaA molecules to ssDUE, the ‘continuous filament’ model (5), or can stabilize DnaA molecules already bound to ssDUE (20), or the polymer of array-bound DnaA molecules itself binds to the ss sites, the ‘ssDUE recruitment’ model (24). The recruitment model appears fully consistent with results of the present studies on ori2 as well as of the past studies on plasmid initiators of RK2 and F (Figure 10) (29).

The DUE binding of initiators might explain another common feature of origin organization: location of the DUE at one end of several origins (Figure 1) (12,13,43). If the opening is induced by torsional stress from ds binding, the opening is likely to diffuse away from DUE, since no barrier to diffusion has been invoked for these origins. Capturing a strand will arrest the opening from diffusing away and keep it localized.

The binding of RctB to ds and ssDUE sites in the ternary complex formation is expected to be reciprocally cooperative. It is known that IHF can promote DnaA binding to weak ds sites in oriC in vivo and in vitro (24,84,85,86). These results can be understood if in the IHF-assisted ternary complex both the ds array and DUE help in each other’s binding to the initiator.
Although DUE has multiple sites, the opening generally initiates at sites closest to the ds array, which will impart a direction to the opening process. For example, of the three 13-mers of *E. coli* oriC, L, M and R, opening requires the R site and is believed to initiate at the R site, the one closest to a ds site array (2,11,85,90,91). In the origins of *B. subtilis* and lambda phage also, the unwinding proceeds from the array of ds sites into DUE (22,26). In ori2, the first two tetramer repeats closest to the ds array are more important for the origin function than the other four repeats and it is where the opening of the origin is most conspicuous (Figures 3 and 9). The positions of these two repeats are also highly conserved relative to the position of 12-mer array and the IHF site (Supplementary Figure S4). Together these results indicate that the proximity to ds sites determines the site preference for initiating the opening. A better understanding of the initiation and propagation of strand opening in any of the replication origins discussed here remains an important challenge.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online.

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**REFERENCES**

1. Leonard, A.C. and Mechali, M. (2013) DNA replication origins. *Cold Spring Harb. Perspect. Biol.*, 5, a010116.
2. Bramhill, D. and Kornberg, A. (1988) Duplex opening by dnaA protein at novel sequences in initiation of replication at the origin of the *E. coli* chromosome. *Cell*, 52, 743–755.
3. Jha, J.K., Ramachandran, R. and Chattoraj, D.K. (2016) Opening the strands of replication origins: still an open question. *Front. Mol. Biosci.*, 3, 62.
4. Wegrzyn, K.E., Gross, M., Uciechowska, U. and Konieczny, I. (2016) Replisome assembly at bacterial chromosomes and iteron plasmids. *Front. Mol. Biosci.*, 3, 39.
5. Duderstadt, K.E. and Berger, J.M. (2013) A structural framework for replication origin opening by AAA+-initiation factors. *Curr. Opin. Struct. Biol.*, 23, 144–153.
6. Fang, L., Davey, M.J. and O’Donnell, M. (1999) Replisome assembly at *oriC*, the replication origin of *E. coli*, reveals an explanation for initiation sites outside an origin. *Mol. Cell*, 4, 541–553.
7. Costa, A., Hood, I.V. and Berger, J.M. (2013) Mechanisms for initiating cellular DNA replication. *Annu. Rev. Biochem.*, 82, 25–54.
8. Douglas, M.E., Ali, F.A., Costa, A. and Diffl ey, J.F.X. (2018) The mechanism of eukaryotic CMG helicase activation. *Nature*, 555, 265–268.
9. Wolanski, M., Donczew, R., Zawilak-Pawlik, A. and Zakrzewska-Czerwinska, J. (2014) *oriC*-encoded instructions for the initiation of bacterial chromosome replication. *Front. Microbiol.*, 5, 735.
10. Leonard, A.C. and Grimwade, J.E. (2005) Building a bacterial orrisome: emergence of new regulatory features for replication origin unwinding. *Mol. Microbiol.*, 55, 978–985.

11. Kowalski, D. and Eddy, M.J. (1989) The DNA unwinding element: a novel, cis-acting component that facilitates opening of the *Escherichia coli* replication origin. *EMBO J.*, 8, 4335–4344.

12. Bramhall, D. and Korndörfer, A. (1988) A model for initiation at origins of DNA replication. *Cell*, 54, 915–918.

13. Rajewski, M., Wegzyn, K. and Konieczny, I. (2012) AT-rich region and repeated sequences: the essential elements of replication origins of bacterial replicons. *FEMS Microbiol. Rev.*, 36, 408–434.

14. Park, K., Mukhopadhyay, S. and Chattoraj, D.K. (1998) Requirements for and regulation of origin opening of plasmid pBR322. *J. Biol. Chem.*, 273, 24906–24911.

15. Roth, A. and Messer, W. (1995) The DNA binding domain of the initiator protein DnaA. *EMBO J.*, 14, 2106–2111.

16. Erzberger, J.P., Pirruccello, M.M. and Berger, J.M. (2002) The structure of bacterial DnaA: implications for general mechanisms underlying DNA replication initiation. *EMBO J.*, 21, 4763–4773.

17. Mukhopadhyay, G. and Chattoraj, D.K. (1993) Conformation of the oriC plasmid replication origin. *Nature*, 401, 296–299.

18. Dederer, K., Emmerich, M., Biondi, C., Ender, A., Krawczyk, A. and Konieczny, I. (2001) Highly organized DnaA–initiator complexes recruit the single-stranded DNA for replication initiation. *Nat. Struct. Biol.*, 8, 1144–1146.

19. Leger, J.F., Romano, G., Sarkar, A., Robert, J., Bourdieu, L., Kemble, H., Bland, M.J., Postoz, C., Koszul, R., Skovgaard, O. and Mazel, D. (2016) A checkpoint control orchestrates the replication of the two chromosomes of *Vibrio cholerae*. *Sci. Adv.*, 2, e1501914.

20. Kemter, F.S., Messerschmidt, S.J., Schlappo, N., Sobotzko, P., Lang, E., Bunk, B., Sproer, C., Teschler, J.K., Yildiz, F.H., Overmann, J. et al. (2018) Synchronous termination of replication of the two chromosomes is an evolutionary selected feature in Vibrio naticae. *PLoS Genet.*, 14, e1007251.

21. Venkova-Canova, T., Chattoraj, D.K. (2012) Evidence for two different regulatory mechanisms linking replication and segregation of *Vibrio cholerae* chromosome II. *PLoS Genet.*, 8, e1002579.

22. Baek, J.H. and Chattoraj, D.K. (2014) Chromosome I controls replication of the second *Vibrio cholerae* chromosome. *Front. Microbiol.*, 5, 1000526.

23. Venkova-Canova, T., Baek, J.H., Fitzgerald, P.C., Blokesch, M. and Chattoraj, D.K. (2013) AT-rich region regulates the initiator protein of *Vibrio cholerae* chromosome II replication. *Proc. Natl. Acad. Sci. U.S.A.*, 110, 10577–10582.

24. Gerding, M.A., Chao, M.C., Davis, B.M. and Waldor, M.K. (2015) Molecular dissection of the essential features of the origin of replication of the second *Vibrio cholerae* chromosome. *mBio*, 6, e00703-15.

25. Pal, D., Venkova-Canova, T., Srivastava, P. and Chattoraj, D.K. (2005) Multipartite regulation of *rctB*, the replication initiator gene of *Vibrio cholerae* chromosome II. *J. Bacteriol.*, 187, 7167–7175.

26. Fekete, R.A., Venkova-Canova, T., Park, K. and Chattoraj, D.K. (2006) IHF-dependent activation of plasmid origin by dnaA. *Mol. Microbiol.*, 62, 1739–1751.

27. Funnell, B.E. (1988) Participation of *Escherichia coli* integration host factor in the Plasmid partition system. *Proc. Natl Acad. Sci. U.S.A.*, 85, 6657–6661.

28. Val, M.E., Skovgaard, O., Ducos-Galand, M., Bland, M.J. and Mazel, D. (2012) Genome engineering in *Vibrio cholerae*: a feasible approach to address biological issues. *PLoS Genet.*, 8, e1002472.

29. Jha, J.K., Demarre, G., Venkova-Canova, T. and Chattoraj, D.K. (2012) Replication regulation of *Vibrio cholerae* chromosome II involves initiator binding to the origin both as monomer and as dimer. *Nucleic Acids Res.*, 40, 6026–6036.

30. Skowyra, D. and Wickner, S. (1993) The interplay of the GrpE heat shock protein and Mg2+ in RepA monomerization by DnaJ and DnaK. *J. Biol. Chem.*, 268, 25396–25401.

31. Schindelin, J., Argandana-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., et al. (2012) Fiji: an open-source platform for biological-image analysis. *Nat. Methods*, 9, 676–686.

32. Venkova-Canova, T., Srivastava, P. and Chattoraj, D.K. (2006) Transcriptional inactivation of a regulatory site for replication of *Vibrio cholerae* chromosome II. *Proc. Natl Acad. Sci. U.S.A.*, 103, 12051–12056.

33. Venkova-Canova, T., Saha, A. and Chattoraj, D.K. (2012) A 29-mer site regulates transcription of the initiator gene as well as function of the replication origin of *Vibrio cholerae* chromosome II. *Plasmid*, 67, 102–110.

34. Stenzel, T.T., Patel, P. and Bastia, D. (1987) The integration host factor of *Escherichia coli* binds to DNA at the origin of replication of the plasmid pSC101. *Cell*, 49, 709–717.

35. Filutowicz, M. and Appelt, K. (1988) The integration host factor of *Escherichia coli* binds to multiple sites at plasmid R6K. *Nature* 332, 3829–3843.

36. Demarre, G. and Chattoraj, D.K. (2010) DNA adenine methylation is required to replicate both *Vibrio cholerae* chromosomes once per cell cycle. *PLoS Genet.*, 6, e1000939.
54. Kimura, T., Asai, T., Imai, M. and Takenami, M. (1989) Methylation strongly enhances DNA bending in the replication origin region of the Escherichia coli chromosome. Mol. Gen. Genet., 219, 69–74.

55. Collins, M. and Myers, R. M. (1987) Alterations in DNA helix stability due to base modifications can be evaluated using denaturing gradient gel electrophoresis. J. Mol. Biol., 198, 737–744.

56. Engel, J. D. and von Hippel, P. H. (1978) Effects of methylation on the stability of nucleic acid conformations. Studies at the polymerase level. J. Biol. Chem., 253, 927–934.

57. Diekmann, S. (1987) DNA methylation can enhance or induce DNA curvature. EMBO J., 6, 4213–4217.

58. Hsu, J., Bramhill, D. and Thompson, C. M. (1994) Open complex formation by DNA initiation protein at the Escherichia coli chromosomal origin requires the 13-mers precisely spaced relative to the 9-mers. Mol. Microbiol., 11, 903–911.

59. Ozaki, S., Kawakami, K., Nakamura, K., Fujikawa, N., Kagawa, W., Park, S. Y., Yokoyma, S., Kurumizaka, H. and Katayama, T. (2008) A common mechanism for the ATP-DnaA-dependent formation of open complexes at the replication origin. J. Biol. Chem., 283, 8351–8362.

60. Brendler, T., Abeles, A. and Austin, S. (1991) Critical sequences in the core of the P1 plasmid replication origin. J. Bacteriol., 173, 3935–3942.

61. Kowalczyk, L., Rajewksa, M. and Konieczny, I. (2005) Positioning and the specific sequence of each 13-mer motif are critical for activity of the plasmid RK2 replication origin. Mol. Microbiol., 57, 1439–1449.

62. Stenzel, T. T., MacAllister, T. and Bastia, D. (1991) Cooperation at a distance promoted by the combined action of two replication initiator proteins and a DNA binding protein at the replication origin of pSC101. Genes Dev., 5, 1453–1463.

63. Kimura, T., Asai, T., Imai, M. and Takanami, M. (1989) Methylation of chromosomal origin requires the 13-mers precisely spaced relative to the replication origin region. Mol. Microbiol., 3935–3942.

64. Goodeman, S. D., Velten, N. J., Gao, Q., Robinson, S. and Segall, A. M. (1999) Differentiation of the DnaA–oriC subcomplex for DNA unwinding in a replication initiation complex. J. Biol. Chem., 274, 30915–30918.

65. Goodman, S. D., Velten, N. J., Gao, Q., Robinson, S. and Segall, A. M. (1999) In vitro selection of integration host factor binding sites. J. Bacteriol., 181, 3246–3255.

66. Stonehouse, E., Kovacicova, G., Taylor, R. K. and Skorupski, K. (2008) Integration host factor positively regulates virulence gene expression in Vibrio cholerae. J. Bacteriol., 190, 4736–4748.

67. Stenzel, T. T., MacAllister, T. and Bastia, D. (1991) Cooperativity at a distance promoted by the combined action of two replication initiator proteins and a DNA binding protein at the replication origin of pSC101. Genes Dev., 5, 1453–1463.

68. Weigel, C., Messer, W., Preiss, S., Welzeck, M., Morigen and Boye, E. (2006) Independent control of replication initiation of the two Vibrio cholerae chromosomes by DnaA and RecB. J. Bacteriol., 188, 6419–6424.

69. Saxena, R., Stanley, C. B., Kumar, P., Cuneo, M. J., Patil, D., Jha, J., Weiss, K. L., Chattoraj, D. K. and Crooke, E. (2020) A nucleotide-dependent oligomerization of the Escherichia coli replication initiator DnaA requires residues His136 for remodeling of the chromosomal origin. Nucleic Acids Res., 48, 200–211.

70. Asai, T., Takenami, M. and Imai, M. (1999) The AT richness and gid transcription determine the left border of the replication origin of the E. coli chromosome. EMBO J., 9, 1008–1015.

71. Hwang, D. S. and Kornberg, A. (1992) Opening of the replication origin of Escherichia coli by DnaA protein with protein HU or IHF. J. Biol. Chem., 267, 23083–23086.