DNA Phase Transition Promoted by Replication Initiator†

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ABSTRACT: DNA is flexible and easily subjected to bending and wrapping via DNA/protein interaction. DNA supercoiling is known to play an important role in a variety of cellular events, such as transcription, replication, and recombination. It is, however, not well understood how the superhelical strain is efficiently redistributed during these reactions. Here we demonstrate a novel property of an initiator protein in DNA relaxation by utilizing a one-molecule-imaging technique, atomic force microscopy, combined with biochemical procedures. A replication initiator protein, RepE54 of bacterial mini-F plasmid (2.5 kb), binds to the specific sequences (iterons) within the replication region (*ori2*). When RepE54 binds to the iterons of the negatively supercoiled mini-F plasmid, it induces a dynamic structural transition of the plasmid to a relaxed state. This initiator-induced relaxation is mediated neither by the introduction of a DNA strand break nor by a local melting of the DNA double strand. Furthermore, RepE54 is not wrapped by DNA repeatedly. These data indicate that a local strain imposed by initiator binding can induce a drastic shift of the DNA conformation from a supercoiled to a relaxed state.

DNA supercoiling results from DNA winding around proteins (as is the case of eukaryotic nucleosome) and/or from the topological constraint imposed upon closed circular DNA (as is the case of bacterial plasmid) and is known to play an important role in a variety of events, such as transcription, replication, and recombination (*1*, *2*). In the case of replication initiation, such supercoiling is necessary for the initiator protein to induce local melting of the DNA double strand (*3*); i.e., the free energy of negative supercoiling is utilized to promote the denaturation of base pairing. Since nearly all of the DNA is negatively supercoiled and highly packaged in cells, such packaging must be promptly rearranged to allow the DNA melting and ensure the proceeding of the reaction. Therefore, there must be a critical mechanism that dynamically affects the superhelical strain of several kilobase long DNA. Despite a number of studies which have demonstrated the importance of superhelical strain in DNA replication, however, poorly understood is how the conformation of long DNA is affected by a local binding of initiator protein.

A number of bacterial plasmids replicate autonomously in host cells. These plasmids have served as a model system to elucidate the molecular basis of replication events, because of their small DNA sizes, simple components of replication machinery, and host-independent mechanism of replication control. The replication of F-plasmid in *Escherichia coli* is initiated by RepE protein (for review, see ref *4*). It binds to the 19 bp direct repeats (iterons) in the replication origin (*ori2*). At the downstream of *ori2* is located a promoter/operator region of the *repE* gene that contains an inverted repeat, half of the sequence of which is similar (8 bp matches) to the iteron. The RepE monomer binds to *ori2* to initiate DNA replication (initiator function), and RepE dimer binds to the promoter/operator region of the *repE* gene to autogenously repress its transcription (repressor function) (*5*, *6*).

To study the molecular mechanism of replication control, a number of F-plasmid derivatives (mini-F) have been constructed. In this study, we investigated the effects of initiator protein on the whole structure of a mini-F plasmid using a one-molecule-imaging technique [atomic force microscopy (AFM)[1]], which is a powerful tool to visualize interactions between proteins and DNA of several kilobases long (*7*). The RepE binding to the iterons was found to drastically convert the plasmid structure from highly supercoiled to relaxed states without changing the linking number. It is most likely that a local binding of RepE and subsequent formation of the nucleoprotein complex at the *ori2* region change the degree of helical twisting of the entire plasmid DNA of several kilobase pairs. This novel mechanism may play an important role in setting a structural environment for the following steps of replication.

EXPERIMENTAL PROCEDURES

Plasmid Construction and Proteins. All plasmids and purified proteins used in this study were described in the

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[1] Abbreviations: AFM, atomic force microscopy; EtBr, ethidium bromide; HEPES, 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; BSA, bovine serum albumin.

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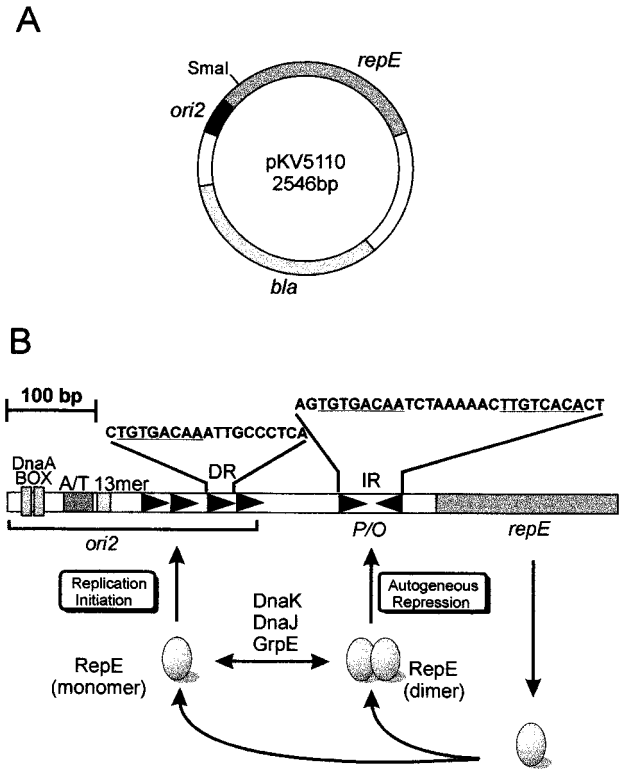

FIGURE 1: (A) Mini-F plasmid used in this study. pKV5110 contains minimal essential components for replication in bacteria (replication origin, *ori2* and *repE* genes). It also carries the *bla* gene as a selection marker. The restriction site for *Sma*I is indicated. (B) Schematic representation of *ori2* and the promoter region of the *repE* gene. DnaA boxes, an A/T-rich region, a 13mer, a direct repeat (DR, iteron), and an inverted repeat (IR) are illustrated. The nucleotide sequences of an iteron and the inverted repeat are also shown (*24*), in which the conserved 8 bp motives are underlined. RepE monomer initiates DNA replication when bound to the iterons, and dimer inhibits the transcription of the *repE* gene when bound to the inverted repeat (*5*). The monomer−dimer conversion of RepE is catalyzed by molecular chaperons, such as DnaJ, DnaK, and GrpE.

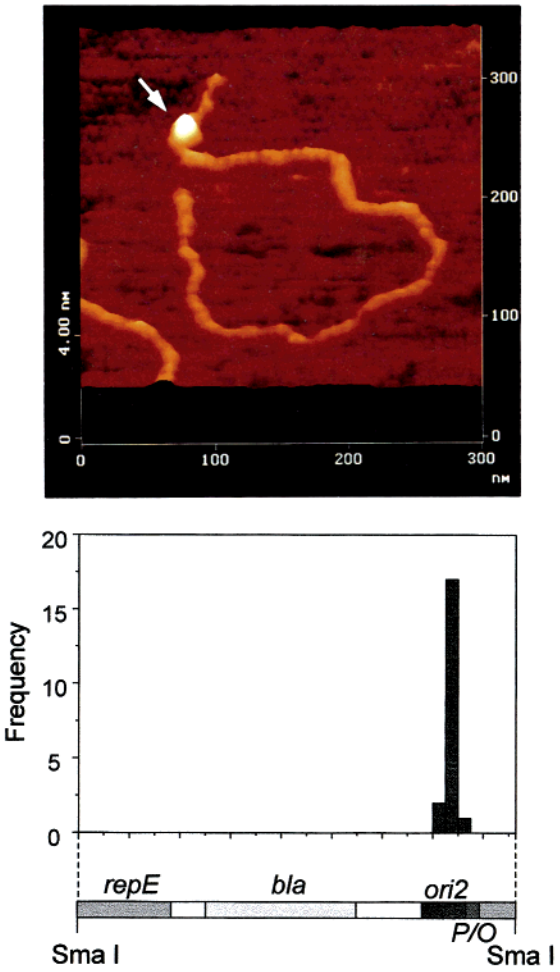

FIGURE 2: Site-specific binding of RepE54 to *ori2*. Purified RepE54 (10 ng) and linearized mini-F plasmid (*Sma*I-digested pKV5110, 5 ng; see Figure 1A) were incubated and then subjected to AFM imaging under the tapping mode. The upper panel shows the AFM image of RepE54 binding to *Sma*I-digested plasmid in a 300 × 300 nm size in a quick surface plot format. Bound protein is indicated with an arrow. The bottom panel shows the frequency plot (histogram) of the protein binding position. The positions of protein bound on the *Sma*I-digested plasmid were determined by measuring the distance from an end of the DNA strand, and the frequency of observations is given in 25 nm bins ($n = 17$). Below the histogram is depicted the map of linearlized pKV5110 in the corresponding position.

previous studies: pKV5110 (*8*), a derivative (pKV7205) of the pBend2 plasmid containing one iteron from *ori2* (*9*), wild-type RepE protein (*10*), and RepE54 protein (*5*). The supercoiled plasmids were purified by the CsCl/EtBr isopicric centrifugation method. The relaxation of the supercoiled plasmid (0.5 μg) was carried out by treating with 1−5 units of topoisomerase I (Promega) in 50 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 1 mM DTT, 20% glycerol, and 50 mM NaCl at 37 °C for 90 min and confirmed by agarose gel electrophoresis.

AFM Imaging and Analysis. The AFM imaging was performed as described previously (*7*). Five to ten nanograms of plasmid DNA (pKV5110 or pKV7205) was incubated with 10−50 ng of proteins (wild-type RepE or RepE54) in 20 μL of 5 mM HEPES buffer (pH 7.4) containing 2 mM $MgCl_2$ at 37 °C for 30 min. The specimen for AFM imaging was prepared by applying the sample (10 μL) on a freshly cleaved mica surface, successive washing with water, and subsequent drying under nitrogen gas. Imaging was carried out using a Digital Instrument Nanoscope III with type E scanner under the tapping mode. AFM tips made of silicon with a cantilever length of 130 nm were used in imaging. The images are collected in the height mode and stored in 512 × 512 pixel format. Obtained images were then plane fitted and analyzed by the computer program accompanied with the imaging module (Digital Instruments). The real dimension of the sample was estimated as described previously (*7*, *11*).

$KMnO_4$ Treatment of the Initiator/DNA Complex. Supercoiled plasmid pKV5110 (1.5 μg) was incubated with various amounts of RepE54 (0, 50, 100, and 500 ng) in 20 mM Tris-HCl (pH 7.5), 40 mM NaCl, 40 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA, and BSA (0.1 mg/mL) in the presence or absence of 0.5 mM ATP at 30 °C for 30 min. $KMnO_4$ was added to a final concentration of 2 mM, and the incubation was continued for an additional 2.5 min. The reaction was terminated by adding β-mercaptoethanol to 250 mM, and the plasmid DNA was purified by passing through Micro Biospin P-30 (Bio-Rad), phenol/chloroform extraction, and subsequent ethanol precipitation.

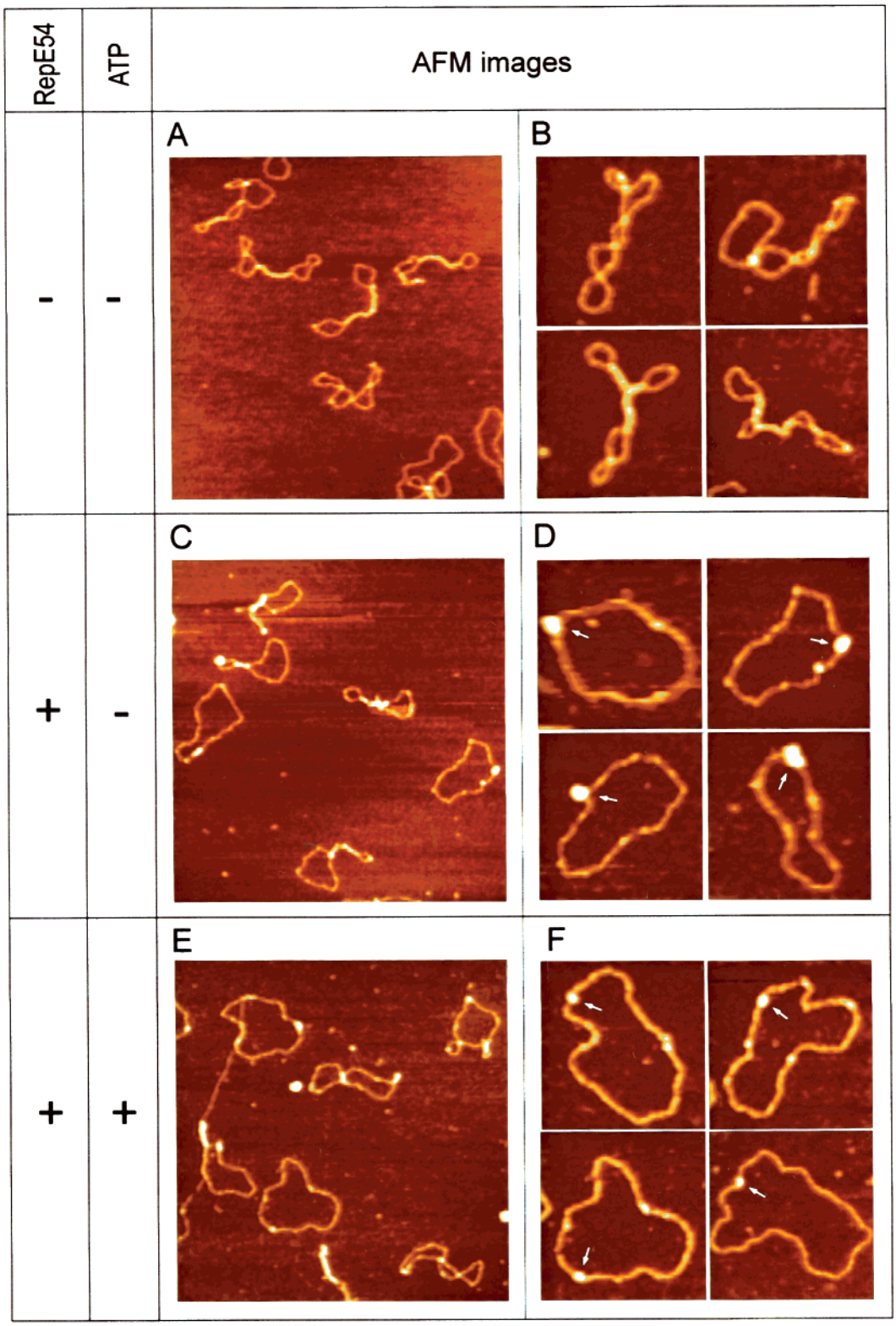

FIGURE 3: RepE54-induced relaxation of mini-F plasmid. Supercoiled plasmid (pKV5110, 5 ng) and RepE54 (10 ng) were incubated in the absence or presence of ATP (10 μM). AFM images of (A and B) supercoiled mini-F plasmids in the absence of RepE54, (C and D) relaxed plasmids by RepE54 binding in the absence of ATP, and (E and F) relaxed plasmids by RepE54 binding in the presence of ATP are shown. The images in F represent the relaxed plasmids only from the longer population (see also Figure 7). Bound proteins are indicated with arrows. Image sizes are 1 $\times$ 1 μm (A, C, and E) and 300 $\times$ 300 nm (B, D, and F) (reproduced at 90% of original size).

The recovered DNA was then treated with 1 M piperidine at 90 °C for 30 min and quenched on ice. The piperidine was removed by repeated lyophilizing and redissolving in water. Using this DNA as a template, primer extension was carried out with ^{32}P-end-labeledprimer (5′-CAACACCT-TCTCTAGAACC-3′, corresponding to the downstream of *ori2*). The reaction products were analyzed on a 6% polyacrylamide/8 M urea sequencing gel.

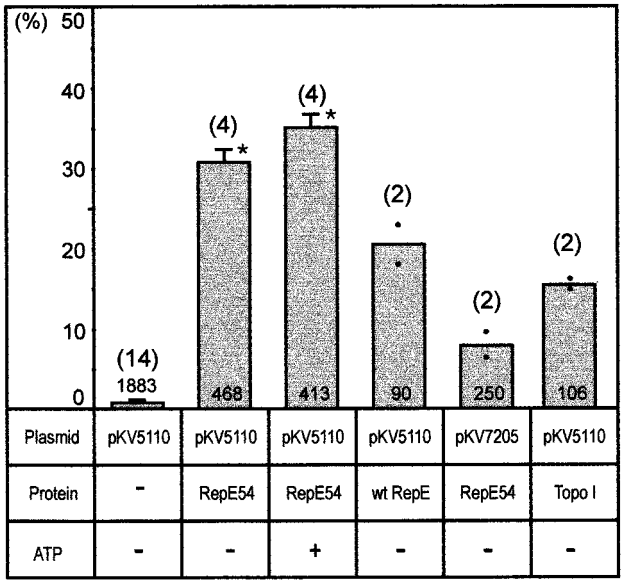

FIGURE 4: Statistical analysis of the relaxation of mini-F plasmids induced in different conditions. The ratio of relaxed plasmid was counted on AFM images as represented in Figure 3. Supercoiled plasmids (pKV5110 or pKV7205) and proteins (wild-type RepE, RepE54, or topoisomerase I) are incubated in the presence (+) or absence (−) of ATP and subjected to AFM imaging. In our AFM analysis, "relaxed form" is defined operationally; only the plasmids visualized without any crossovers are considered as relaxed. This means that even a completely relaxed plasmid ($\Delta Lk = 0$) may be recognized as a tangled one if it shows crossover during the specimen preparation due to mechanical (physical) constraint on mica surface. Bars represent standard errors of 4−14 independent experiments, and the number of experiments is shown in parentheses. Each experiment consisted of 40−150 different observations, and the total number of observations is indicated. The asterisk (*) indicates that the differences between with and without RepE54 are significant ($p < 0.001$).

RESULTS

In this study, one of the mini-F plasmid derivatives that contains the minimal essential components for replication (pKV5110, Figure 1A) was used. Figure 1B illustrates the replication origin (*ori2*) of mini-F plasmid, which contains two DnaA boxes, an A/T-rich region, and four iterons. Since RepE is considerably stable as a dimer (*5*), we utilized a mutant protein that is stable as a monomer with higher initiator activity (RepE54; *12*) to investigate its functional involvement in the replication initiation.

RepE54 Preferentially Binds to Iterons and Causes Relaxation of Mini-F Plasmid. The binding of RepE54 to the mini-F plasmid, pKV5110, and its structural effect were visualized using AFM. First, we confirmed the site-specific binding of RepE54 to the iterons within *ori2* (Figure 2). The binding site was determined by measuring the distance from an end of the linearized DNA. This result is consistent with the previous finding of gel shift assay that the RepE monomer preferentially binds to the iterons (*13*).

The structure of pKV5110 freshly isolated from bacteria was visualized under AFM. Almost all of the plasmids were observed as highly tangled forms (Figure 3A), consistent with the notion that almost all plasmids are highly negatively supercoiled in cells (*22*). A statistical analysis of the obtained images revealed that the plasmids without any crossovers (i.e., in a relaxed circular form) were less than 2% of the total plasmids (Figure 4). In this series of experiments, "relaxed" plasmid is defined operationally as the one lacking any crossovers of DNA strand on AFM images.

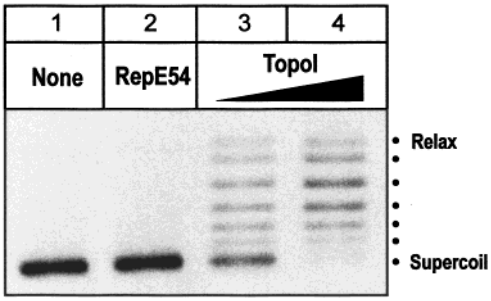

FIGURE 5: RepE54 does not have topoisomerase-like activity. The supercoiled mini-F plasmid (pKV5110, 0.5 μg) was incubated at 37 °C in the absence (lane 1) or in the presence (lane 2) of RepE54 (0.1 μg). The plasmid (0.5 μg) was treated with 0.5 unit (lane 3) or 1 unit (lane 4) of topoisomerase I. After phenol/chloroform extraction, the topology of the plasmid DNA was analyzed by agarose gel electrophoresis. The positions of topoisomers are indicated with dots. The linking number change (ΔLk) of this plasmid is determined to be ∼−7.

When these supercoiled plasmids were subjected to the binding of RepE54, the population of the relaxed circular form was drastically increased to 30% (Figures 3B and 4). This number is remarkably high, considering our operational definition of relaxation (see the legend for Figure 4). Note that only 16% of the topoisomerase I-treated pKV5110 was in a relaxed form under AFM (Figure 4). Wild-type RepE could induce relaxation, but to a smaller extent (Figure 4), consistent with the fact that the wild-type RepE exists mostly as a dimer (*5*). These results indicate that the binding of the RepE monomer (but not the RepE dimer) to iterons in *ori2* causes plasmid relaxation. Since only 8% of the plasmid bearing only one iteron (pKV7205; Figure 4) was relaxed and the plasmid lacking iteron (pUC18) was not relaxed (<1%; data not shown) by RepE54, multiple iterons are required for the dynamic structural change of the plasmid DNA.

Initiator-Induced Plasmid Relaxation Does Not Result from the Linking Number Change. A plausible explanation for the RepE54-induced plasmid relaxation is that RepE54 has a topoisomerase activity like T-antigen of SV40 (*14*) and changes the linking number of the closed circular plasmid by introducing a strand break. Although such a topoisomerase-like activity of RepE has not been reported previously, we reevaluated the ability of RepE54 to catalyze a topological change of supercoiled plasmid. However, no such activity was detected when pKV5110 was incubated with RepE54 and analyzed on agarose gel electrophoresis (Figure 5, lane 2). The linking number change (ΔLk) of the native supercoiled mini-F plasmids (2.5 kb) was determined to be ∼−7 by mildly treating the plasmid with topoisomerase I (Figure 5, lanes 3 and 4). Thus, the relaxation of the supercoiled plasmid upon RepE54 binding does not result from the removal of superhelical strain by introducing a strand break.

RepE54 Does Not Induce DNA Melting at ori2. The other possible mechanism which could reduce the negative superhelical strain without changing the linking number is local melting of the DNA double strand. To examine whether RepE protein induces double strand melting around the *ori2*

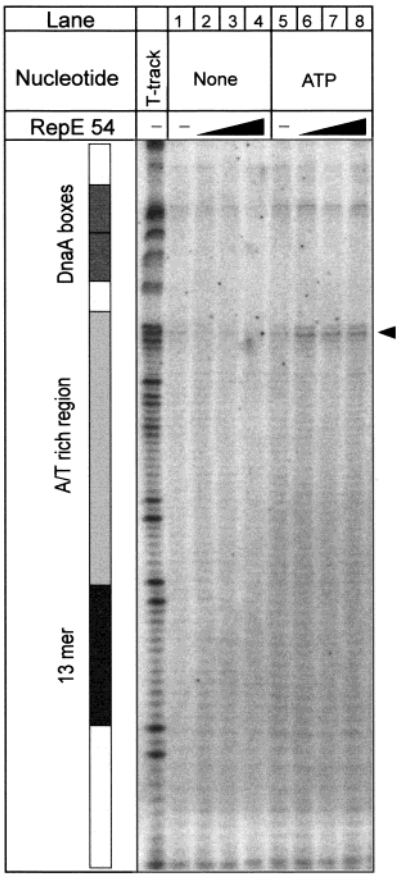

FIGURE 6: RepE54 does not induce DNA melting within *ori2*. Local melting of the DNA double strand induced by RepE54 was investigated by $KMnO_4$ assay. Supercoiled pKV5110 and RepE54 were incubated in the absence (lanes 1−4) or presence (lanes 5−8) of 0.5 mM ATP and treated with $KMnO_4$. The corresponding position in *ori2* is depicted in the left.

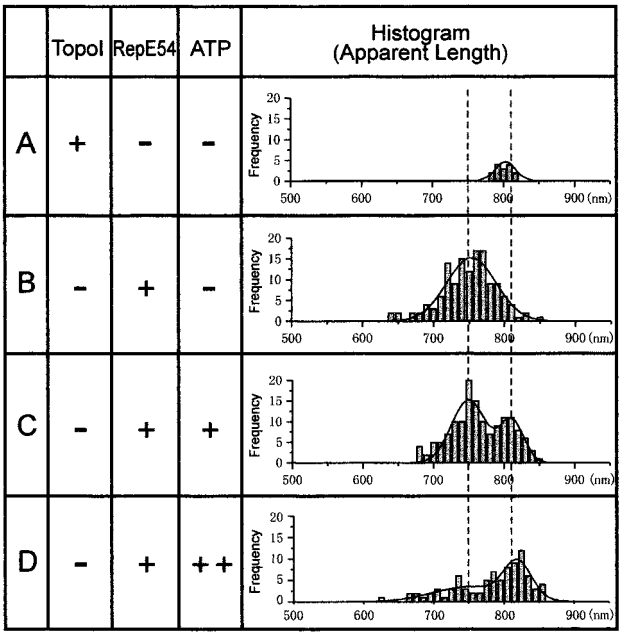

FIGURE 7: The RepE-induced relaxed state of the mini-F plasmid is concomitant with the reduction of an apparent plasmid length. The apparent lengths of mini-F plasmid relaxed under various conditions were measured and histogramed in 10 nm bins. The experimental conditions are as follows: (A) in the presence of topoisomerase I (control for complete relaxation), (B) in the presence of RepE54 only, (C) in the presence of RepE54 and 10 μM ATP, and (D) in the presence of RepE54 and 1 mM ATP. Solid lines are obtained by Gaussian fitting, and the mean values of each peak are as follows: (A) 798 ± 28 nm (mean ± SE) (the total number of observations, n = 17), (B) 753 ± 17 nm (n = 137), (C) 750 ± 24 and 811 ± 17 nm (n = 144), and (D) 751 ± 19 and 819 ± 50 nm (n = 87). The values for (B) − (D) were obtained from three to four independent experiments, and each experiment consisted of 20−40 different observations. The positions of 750 and 810 nm are also indicated with vertical dotted lines.

region, a chemical probe, i.e., $KMnO_4$, was utilized (*15*). The supercoiled pKV5110 and RepE54 were preincubated and then treated with $KMnO_4$. As shown in Figure 6, RepE54 did not cause any specific melting of the double helix within the *ori2* region (lanes 1−4). An addition of HU protein indeed stimulated the melting of over 30 bp in the A/T-rich region (data not shown), consistent with the result in the previous study (*9*). In conclusion, RepE54 itself induces no DNA melting around the *ori2* region.

RepE54 Traps a Very Short Stretch of DNA into a Nucleoprotein Complex. Alternatively, the binding of RepE54 may form a nucleoprotein complex which absorbs the negative supercoiling by DNA wrapping. This possibility was tested by detailed geometrical characterization of the AFM images of relaxed plasmids. The topoisomerase I-treated pKV5110 was used as a control for complete relaxation, whose length was 798 ± 28 nm (Figure 7A). The apparent length of the RepE54-induced relaxed plasmid was significantly shorter, 753 ± 17 nm (Figure 7B). This shortening by RepE54 was not observed when linearized plasmids were used (Figure 2), indicating that RepE54-induced DNA incorporation requires superhelical strain.

The reduction in the apparent length (45 nm) should be caused by an incorporation of a stretch of DNA (corresponding to ~140 bp) into the nucleoprotein complex. In consistency with this notion, the size of the DNA/RepE complex [>11 nm in diameter after considering the curvature of the scanning tip (*7, 11*)] is much larger than the RepE54 tetramer (9.1 nm), which is estimated from a crystalographical study (*16*). Considering that four RepE54 proteins bind to iterons and form a nucleoprotein complex with simple DNA wrapping, the 45 nm stretch of the DNA strand could supply the RepE54 tetramer with only about 1.2 turns, which seems too small an effect to cancel out the superhelical strain ($\Delta Lk \cong -7$). The details of this issue will be addressed in the Discussion.

Plasmid Relaxation by RepE54 in the Presence of ATP. In the replication of the *E. coli* chromosome, an initiator protein, DnaA, binds to each of the five repeats of the 9 bp sequence (DnaA box) within the replication origin, *oriC* (*17*), and forms an open complex at the adjacent A/T-rich region in an ATP-dependent manner (*18*). In the case of simian virus 40 (SV40), an early gene product, large T-antigen, recognizes inverted pairs of a specific binding motif, GAGGC, in the replication origin (*19*) and induces DNA melting at the adjacent palindrome element and DNA distortion at the adjacent A/T-rich region in an ATP-dependent manner (*20*). An origin recognition complex (ORC) of budding yeast is also qualified as a replication initiator that has an ATP-dependent DNA binding activity (*21*). Thus, ATP-dependent DNA binding and subsequent formation of a nucleoprotein complex, involving DNA melting and/or unwinding, seem to be common functions of initiator proteins. Therefore, we

investigated the effect of ATP on the higher order structural change of a supercoiled plasmid induced by RepE54.

In the presence of ATP, 35% of the plasmids were relaxed by RepE54 (Figures 3C and 4), which is slightly higher than that in the absence of ATP. In this condition, however, the apparent length of the RepE54-induced relaxed plasmid had two populations; in addition to the population with an apparent length of 750 ± 24 nm, another population with an apparent length of 811 ± 17 nm appeared (Figure 7C). It should be noted that the peak value of the shorter population (750 nm) was almost equal to that of the RepE54-treated plasmid in the absence of ATP (753 nm) and that of the longer population (811 nm) was almost the same as the topoisomerase-treated plasmid (798 nm). When the concentration of ATP was increased to 1 mM, the longer population became the major one (Figure 7D). These results suggest that only the shorter population, but not the longer one, is associated with DNA incorporation. Indeed, whereas the size of the DNA/RepE complex in the absence of ATP was much larger than that of the RepE tetramer due to DNA incorporation as shown in the previous section, the size of the nucleoprotein complex in the presence of ATP was almost equal to that of the RepE tetramer (Figure 3F).

In the presence of ATP, no significant melting of the DNA double strand within *ori2* was detected by $KMnO_4$ assay (Figure 6). Only a very weak RepE-specific melting (~3 bp) was detected at the edge of the A/T-rich region (Figure 6, lanes 5−8; see arrowhead). However, when we consider the effect of DNA melting on the superhelical structure of the plasmid, the melting of 3 bp has little effect on the superhelical structure of the 2.5 kbp plasmid. Thus, it seems also unlikely that the mere melting of the DNA double strand mediated by RepE54 causes the complete relaxation of the mini-F plasmid.

DISCUSSION

The relaxation of a closed circular plasmid could be caused by several mechanisms mediated by the binding of a certain protein(s). (1) At least one of the DNA strands is cut and, after the superhelical strain is removed, resealed by a specific protein such as topoisomerase. (2) The DNA strand wraps around a protein, as is the case of a nucleosome, so that the superhelical strain is canceled out. (3) The binding of a protein induces a local melting of the DNA double strand at an adjacent region. In the case of the RepE54-induced relaxation, however, none of these mechanisms should be the case. RepE54 could neither catalyze the linking number change (Figure 5) nor catalyze DNA melting (Figure 6). It only mediates the formation of a small nucleoprotein complex with a very short stretch of DNA incorporated (Figure 7). A statistical analysis revealed that a mere wrapping was not enough to promote the complete relaxation by absorbing the superhelical strain; supposing that the DNA wraps around the RepE tetramer in a left-handed manner, 45 nm DNA (corresponding to ~140 bp; Figure 7) could cancel out only +1.2 of the entire negative supercoiling ($\Delta Lk \cong -7$; Figure 5). Therefore, there must be other mechanisms which govern the initiator-induced relaxation of a supercoiled plasmid.

Novel Mechanism of Structural Transition of Plasmid DNA Induced by Local Initiator Binding. The topological property of a closed circular plasmid is described by a mathematical parameter, linking number (*Lk*). This linking number can be represented as a sum of the two geometrical parameters: twisting number (*T*) and writhing number (*W*) ($Lk = T + W$). The twisting number stands for the number of twistings of one single strand of the DNA over the other and depends on both the pitch length and the total number of base pairs. The writhing number stands for how many times the DNA double strand coils and, thus, represents the apparent number of supercoilings. When the linking number of the purified mini-F plasmid is represented as $Lk_0 = T_0 + W_0$, W_0 is significantly negative (~−7). This is because the plasmid freshly purified from bacteria is negatively supercoiled (*22*) and indeed appears highly tangled on AFM images (Figure 3A). Upon RepE54 binding, the total linking number of the plasmid ($Lk_R = T_R + W_R$) remains constant ($Lk_0 = Lk_R$; i.e., $T_0 + W_0 = T_R + W_R$), since RepE54 does not have topoisomerase activity. When a supercoiled plasmid is relaxing in the presence of RepE54, the writhing number of the plasmid (W_R) is reaching 0, and at the same time, the twisting number (T_R) would be decreasing from T_0 to $T_0 + W_0$. Namely, the simplest explanation for the mechanism of RepE-induced relaxation is that the superhelical strain of a closed circular plasmid can be drastically redistributed over several kilobases from writhing to twisting upon protein binding (Figure 8) and which, in turn, induces an apparent relaxation.

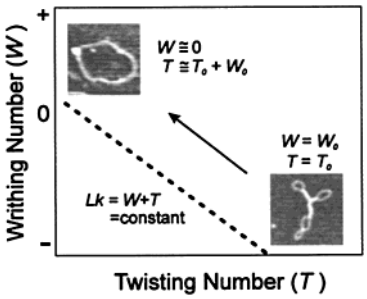

FIGURE 8: Relationship between the twisting number and the writhing number at a constant linking number. The linking number (*Lk*) is expressed as a sum of the twisting number (*T*) and the writhing number (*W*). Without any strand break or nicking, the linking number remains constant (dotted line), and thus, the superhelical strain is distributed between twisting and writhing. The local binding of RepE54 dramatically shifts the distribution from writhing to twisting and promotes a plasmid relaxation (arrow).

Based on the theoretical modeling, Monte Carlo simulation (*23*), a given DNA strand is predicted to be rather flexible and can absorb superhelical strain to a limited degree without changing the apparent shape (up to 1 helical turn per ~150 bp). However, when the helical strain becomes larger than this capacity, the apparent structure of the DNA strand is drastically changed to form supercoiling. This means that when the torsional strain is near this border, a subtle change of the strain could induce a sudden conformational change of the DNA between supercoiled and relaxed states (Figure 8). Thus, the local strain of the DNA strand induced by a specific binding of initiator protein could cause a drastic redistribution of the superhelical strain within the entire plasmid and lead to a dynamic phase transition of the DNA structure. This physical property of DNA must play an important role in a dynamic structural transition of a DNA strand upon replication initiation, as well as in a transcriptional regulation and recombination.

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