Transcriptional Repressor TrmBL2 from *Thermococcus kodakarensis* Forms Filamentous Nucleoprotein Structures and Competes with Histones for DNA Binding in a Salt- and DNA Supercoiling-dependent Manner*

Artem K. Efremov†‡§∗, Yuanyuan Qu†‡§∗, Hugo Maruyama†‡§∗, Ci J. Lin†‡§‡‡, Kunio Takeyasu§∗, and Jie Yan†‡§‡‡

*From the †Mechanobiology Institute, National University of Singapore, Singapore 117411, Singapore, the ‡Centre for Bioimaging Sciences, National University of Singapore, Singapore 117546, Singapore, the §Department of Physics, National University of Singapore, Singapore 117542, Singapore, the ¶School of Physics, Shandong University, Jinan 250100, China, the **Department of Bacteriology, Osaka Dental University, Hirakata 573-1121, Japan, the ††National University of Singapore Graduate School for Integrative Sciences and Engineering, Singapore 119077, and the ‡‡§ Laboratory of Plasma Membrane and Nuclear Signaling, Graduate School of Biostudies, Kyoto University, Kyoto 606-8501, Japan*

**Background:** TrmBL2 and histones maintain the genome functioning in hyperthermophilic euryarchaeal cells.

**Results:** TrmBL2 forms filaments on DNA through cooperative binding and competes with histones in a salt- and DNA supercoiling-dependent manner.

**Conclusion:** TrmBL2-histone collective behavior dynamically controls DNA organization.

**Significance:** The discovered mechanisms provide insights into the regulation of the genome structure and global transcription profile by TrmBL2 and histones.

Architectural DNA proteins play important roles in the chromosomal DNA organization and global gene regulation in living cells. However, physiological functions of some DNA-binding proteins from archaea remain unclear. Recently, several abundant DNA-architectural proteins including histones, Alba, and TrmBL2 have been identified in model euryarchaeon *Thermococcus kodakarensis*. Although histones and Alba proteins have been previously characterized, the DNA binding properties of TrmBL2 and its interplay with the other major architectural proteins in the chromosomal DNA organization and gene transcription regulation remain largely unexplored. Here, we report single-DNA studies showing that at low ionic strength (<300 mM KCl), TrmBL2 binds to DNA largely in non-sequence-specific manner with positive cooperativity, resulting in formation of stiff nucleoprotein filamentous patches, whereas at high ionic strength (>300 mM KCl) TrmBL2 switches to more sequence-specific interaction, suggesting the presence of high affinity TrmBL2-filament nucleation sites. Furthermore, *in vitro* assays indicate the existence of DNA binding competition between TrmBL2 and archaeal histones B from *T. kodakarensis*, which can be strongly modulated by DNA supercoiling and ionic strength of surrounding solution. Overall, these results advance our understanding of TrmBL2 DNA binding properties and provide important insights into potential functions of architectural proteins in nucleoid organization and gene regulation in *T. kodakarensis*.

Organizational and functional maintenance of the compact structure of the chromosomal DNA inside living cells is accomplished through an intricate interplay of several key processes, whose main elements are DNA supercoiling (1–3), macromolecular crowding (2, 4–6), and DNA-architectural proteins (7, 8). Although macromolecular crowding and DNA supercoiling cause nonspecific condensation of the chromosomal DNA, it is known that accessibility of the genome to a plethora of DNA-binding proteins is critical to maintaining its proper function as a dynamic structure capable of rapid reorganization in response to environmental changes (9–11). Thus, besides DNA compaction, the main role of DNA-architectural proteins is to support a delicate balance between the chromosomal DNA condensation and accessibility levels.

To understand the chromosomal DNA organization, it is crucial to know the binding properties and physiological functions of DNA-architectural proteins. Although extensively investigated in bacterial and eukaryotic cells (8, 10–12), much less is known about the genome architecture and physiological roles of DNA-structuring proteins in archaea cells, which form the third domain of life. Until recently, only two abundant DNA-structuring protein families had been identified as widespread among euryarchaeon cells, one of the two phyla of the archaeal domain. It is now well established that these families, histones and Alba proteins (13), contribute to DNA condensation (14–16) and to DNA bridging (17, 18), respectively. More recently, another abundant DNA-architectural protein, TrmBL2, a 30.8-kDa protein with a helix-turn-helix motif typical for transcription factor and regulator proteins (19), was found in model euryarchaeon *Thermococcus kodakarensis* (20). Given its relatively new discovery, the role of TrmBL2 in the genome organization and regulation is not yet well established.

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†To whom correspondence may be addressed. Tel. and Fax: 81-75-753-6852, E-mail: takeyasu@lif.kyoto-u.ac.jp.
‡To whom correspondence may be addressed. Tel.: 65-6515-2620; Fax: 65-6777-6126; E-mail: phyyj@nus.edu.sg.

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Consistent with its structure, our previous data indicate that TrmBL2 functions as a global repressor by regulating the transcription level of ~6.5% of the genes in T. kodakarensis cells (151 of 2306 identified reading frames) via formation of nucleoprotein filamentous structures on the chromosomal DNA (20). However, aside from this, little is known about physicochemical properties of TrmBL2 and its interplay with other architectural proteins in the chromosomal DNA organization. Studying these questions is not only crucial for understanding molecular mechanisms responsible for maintenance of the genome functionality in hyperthermophilic euryarchaeons, but, more importantly, for gaining insights into physical processes governing emergent collective behavior of DNA-architectural proteins in living cells. Therefore, we examined the DNA binding properties of TrmBL2, as well as its impact on DNA supercoiling and its cooperation with archael histones in DNA shaping using a combination of single-DNA magnetic tweezers manipulation techniques and atomic force microscopy (AFM)3 imaging.

Our results suggest that, in contrast to histones and Alba proteins, TrmBL2 antagonizes packaging of DNA by forming stiff nucleoprotein filamentous structures. Moreover, it was found that TrmBL2 filaments can modulate the physical organization of DNA by competing with histones for binding to overlapping nucleotide sequences in a salt- and DNA supercoiling-dependent manner. Thus, in addition to gene repression, TrmBL2 likely acts as a regulator of the chromosomal DNA condensation level, a function that may further impact the global transcription profile. Based on our experimental data, we propose a working mechanism for TrmBL2, by which it may regulate the nucleoid structure and the global gene expressions level in response to environmental cues. Together, these results provide important insights into potential physiological functions of TrmBL2 and archael histones in the chromosomal DNA organization and gene regulation in T. kodakarensis cells.

Materials and Methods

Recombinant Protein Expression and Purification—Recombinant proteins were expressed and purified according to the previously published protocol (20) with modifications. T. kodakarensis open reading frames encoding TrmBL2 (TK0471) or histone B (HTkB; TK2289) were cloned into pET vectors (Novagen) with no additional amino acid sequence added to the native protein sequence (pET-TK0471 and pET-HTkB). Escherichia coli BL21-CodonPlus (DE3)-RII competent cells (Agilent technologies) were transformed with these plasmids and expressed in E. coli. The protein solution was loaded on a HiTrap heparin HP column. The protein was eluted in a linear 50–600 mM KCl gradient in 50 mM Tris- HCl (pH 8.0). Peak fractions containing HTkB were pooled and concentrated with Vivaspin 20 (100,000 molecular weight cut-off). The purity of TrmBL2 preparation estimated by SDS-PAGE gel analysis was found to be ~85% (data not shown). Cells expressing HTkB were resuspended in lysis buffer H (50 mM Tris- HCl, pH 8.0, 100 mM NaCl) and disrupted by sonication. E. coli protein was removed as described above, and the supernatant was incubated with 5 units/ml DNase I for 2 h at 37 °C in the presence of 8 mM MgCl2. The mixture was dialyzed against 50 mM Tris-HCl (pH 8.0) for 16 h, resulting in the formation of HTkB precipitate. The precipitate was dissolved by adding NaCl to 200 mM. The protein solution was loaded on a HiTrap heparin HP column (GE Healthcare) pre-equilibrated with 50 mM Tris-HCl (pH 8.0) and 200 mM NaCl. Recombinant HTkB was eluted in a linear 200–800 mM NaCl gradient in 50 mM Tris-HCl (pH 8.0). Peak fractions containing HTkB were pooled and concentrated with Vivaspin 6 (5000 molecular weight cut-off). The concentration of HTkB was determined with L-8900 amino acid analyzer (Hitachi High-Technologies).

Intracellular Protein Concentration Measurements—To estimate the intracellular concentration of TrmBL2, the total amount of this protein per 1 ml of culture adjusted to A660 = 0.5 was measured using quantitative Western blotting (21) and found to be equal to 2.7 ± 0.5 μg (n = 6). The total cell volume at these conditions was determined by weighting a cell pellet harvested from 18 ml of culture of T. kodakarensis KOD1 and was found to be equal to 50 mg at A660 = 0.593, which is equivalent to ~2.4 mg of the cell pellet per 1 ml of KOD1 culture at A660 = 0.5. Because the density of the cells is very close to that of water (i.e. ~1 g/ml), the obtained pellet mass corresponds to the total cell volume of 2.4 μl. From the measured amount of TrmBL2 (2.7 ± 0.5 μg), its molecular mass (30.8 kDa) and the total volume of cells in the culture (2.4 μl) the intracellular concentration of TrmBL2 was estimated to be 37 ± 7 μM.

Transverse Magnetic Tweezers Experiments—The magnetic tweezers setup used in this study is similar to that previously reported (22, 23). Briefly, a flow channel containing a thin #0 glass coverslip with a streptavidin-coated polished edge was assembled and incubated with 2% BSA solution in 1× PBS overnight at 4 °C prior to experiment. On the day of the experiment, λ-DNAs (48,502 bp; New England Biolabs) labeled with biotin on the both ends were washed into the channel at the concentration of 0.2 ng/μl in 1× PBS and incubated for 10 min at room temperature. After that, 200 μg/ml of 2.8-μm-sized streptavidin-coated magnetic beads (Dynabeads M-280 streptavidin; Invitrogen) were added into the channel and incubated for another 10 min. Unbound λ-DNAs and magnetic beads were then removed from solution by washing the channel with working buffer (10 mM Tris, pH 7.5, 50 mM KCl for Figs. 2A and 7C, 300 mM KCl for Fig. 7D, and 50–600 mM KCl for Fig. 3). Single

3 The abbreviations used are: AFM, atomic force microscopy; HTkB, histone B from T. kodakarensis; RNP, RNA polymerase.
DNA molecules that were bound to the coverslip edge on one end and to a magnetic bead on the other were stretched in the microscope focal plane using a pair of Neodymium magnets mounted onto a translational micromanipulator (MP-285; Sutter Instruments). Force extension curves of each λ-DNA tether were plotted by measuring the DNA extension at different applied loads via tracking the distance between the bead and coverslip edge for 30 s at a 400-Hz rate using home-written software in Labview. All magnetic tweezers experiments were conducted at 25°C.

**DNA Preparation for Supercoiling Experiments**—For DNA supercoiling experiments, we used nick-free 7-kilobase pair DNA constructs including 1-kilobase pair biotin- and digoxigenin-labeled handles, which were prepared according to the following procedure. The two handles and the central part of the construct were obtained through PCR of the A-DNA template, where the handle labeling was done by using biotin-16-dUTP and digoxigenin-11-dUTP nucleotides (Roche) in the respective amplification reactions. Purified biotin- and digoxigenin-labeled handles and the central part of the construct were digested by NsiI and MluI enzymes (New England Biolabs). All three DNA pieces were ligated by incubating them with T4 ligase overnight at 16°C.

**Supercoiling Magnetic Tweezers Experiments**—In DNA supercoiling experiments, we used a simple flow channel (40–50 μl of volume) prepared from two 1 glass coverslip, which were spaced by two paraffin strips. The bottom coverslip was functionalized with Silane-PEG-NHS (PG2-NSSL-10k, Nanocs) (24) and further covered with anti-digoxigenin Fab fragments (Roche) creating covalent bonds with NHS group on PEG. To avoid nonspecific binding of DNA to the surface of the flow chamber, the latter was filled with 2% BSA solution in 1× PBS buffer and incubated overnight at 4°C before the experiment.

To create torsion constrained DNA tether in the flow chamber, preliminary prepared nick-free DNA constructs with biotin- and digoxigenin-labeled handles were washed into the chamber at 0.1–0.2 ng/μl concentration in 1× PBS buffer and incubated for 10 min at room temperature. Following incubation, 50 μg/ml of 1 μm sized streptavidin-coated magnetic beads (Dynabeads MyOne streptavidin; Invitrogen) were added into the chamber and incubated for another 10 min, allowing them to attach to the DNA constructs. Unbound DNA constructs and magnetic beads were then removed from solution by the chamber washing with working buffer (10 mM Tris, 50 mM KCl, pH 7.5). Experiments were conducted at 25°C.

For DNA supercoiling, we used the magnetic tweezers setup similar to that previously described (25). Briefly, a pair of Neodymium magnets was mounted onto a translational micromanipulator (MP-285; Sutter Instruments) and a rotation stage (M660; Physik Instruments) allowing achievement of a full three-dimensional control of the magnets position/rotation angle. Winding/unwinding of torsion-constrained DNA tethers attached to magnetic beads was done by rotating the magnet in clockwise/counter-clockwise direction, respectively. Positions of the tethered beads were tracked with 5-nm spatial resolution at 80 Hz using a home-written bead three-dimensional tracking software (see Ref. 26 for more details). Twist extension curves of a mechanically stretched supercoiled DNA constructs were plotted by measuring the DNA extension for 20 s at different magnet turns.

To convert the magnet turns into a more descriptive physical quantity, we used a notion of the DNA superhelical density. From the existing experimental studies, it is known that DNA has a double-helix structure. The number of times the two strands of DNA are twisted around each other is known as the linking number, Lk. Because the step size of a double-helix DNA in physiologically relevant B-conformation is 10.5 bp/turn, the linking number of a torsion-relaxed DNA having N base pairs is \( L_k = N/10.5 \). Then the superhelical density (also referred to as linking number density/specific linking difference) of a DNA that was overwound/underwound by M turns is defined as \( \sigma = (L_k - L_{k_0})/L_{k_0} = M/L_{k_0} \). Negative/positive \( \sigma \) values refer to negatively (underwound) or positively (overwound) supercoiled DNA, respectively. This number simply shows how many turns are removed/added into the DNA per a single turn of the two strands in the relaxed configuration.

**Atomic Force Microscopy**—To minimize the surface influence on the structure of TrmBL2- and histone-DNA complexes in our AFM experiments, we used glutaraldehyde-coated mica, which better preserves the native conformation of protein-DNA samples compared with freshly cleaved or (3-aminopropyl)triethoxysilane-modified mica (27, 28). To prepare it, freshly cleaved mica was treated with 0.1% (3-aminopropyl)triethoxysilane solution for 15 min, then rinsed with deionized water, dried with nitrogen gas, and incubated in a desiccator overnight. On the next day, 1% glutaraldehyde solution was added to the mica surface for 15 min to allow glutaraldehyde molecules covalently bind to it. After the incubation, the mica was again rigorously washed with deionized water and dried with nitrogen gas. Protein-DNA samples were made by preparing solution of 0.2 ng/μl, 1039-bp DNA with TrmBL2 (6, 20, or 60 nm) or HCKB (0.6 nm) in working buffer (10 mM Tris, pH 7.5, 50 mM KCl for Figs. 1 and 7, A and B; 300 mM KCl for Fig. 4A; and 600 mM KCl for Fig. 4B) and incubating it for 30 min at 25°C/70°C. After that, it was immediately deposited on a glutaraldehyde-mica for 10 min. The complexes that did not attach to the glutaraldehyde surface were washed away with 3 ml of deionized water, and the mica was dried with nitrogen gas. Imaging of the prepared samples was done using an AFM (Fast-Scan, Bruker, Singapore) in Acoustic AC mode at 512 × 512 pixels resolution (pixel size = 3.9 nm) and scan speed of 2 lines per second. Acquired AFM images of DNAs were analyzed utilizing the centerline tracing algorithm (29).

**Occupancy Ratio and Data Fitting**—Formation of TrmBL2 nucleoprotein filaments on DNA causes its stiffening by increasing the effective persistence length, \( A_{DNA-TrmBL2} \). Measuring \( A_{DNA-TrmBL2} \) as a function of the protein concentration, \( c \), and by using the worm-like chain model of DNA (30), it is easy to calculate the fraction of DNA binding sites occupied by TrmBL2 proteins, \( \alpha(c) \), as (18, 31) follows.

\[
\alpha(c) = \frac{1}{\frac{A_{DNA-TrmBL2}(c)}{A_{naked-DNA}}} - \frac{1}{\frac{A_{saturated-DNA}}{A_{naked-DNA}}} \quad \text{(Eq. 1)}
\]
Here \( A_{\text{naked-DNA}} = A_{\text{DNA-TRmBL2}}(c = 0) \) is the persistence length of a naked DNA, and \( A_{\text{saturated-DNA}} = A_{\text{DNA-TRmBL2}}(c \to \infty) \) is the effective persistence length of a DNA with all binding sites occupied by TRmBL2 proteins.

In general, the binding cooperativity, \( n \), and dissociation equilibrium constant of a protein, \( K_d \), can be estimated by fitting the DNA occupancy fraction to the famous Hill equation.

\[
\alpha(c) = \frac{c^n}{c^n + K_d^n}
\]

(Eq. 2)

However, as was found in our study, TRmBL2 has two concentration-dependent binding regimes to DNA, which cannot be well described by the above Hill equation (Fig. 2C). To account for them, experimental data points were fitted to the double Hill equation instead.

\[
\alpha(c) = \frac{c^{n_1}}{c^{n_1} + K_{d1}^{n_1}} + \left(1 - \frac{c^{n_2}}{c^{n_2} + K_{d2}^{n_2}}\right)
\]

(Eq. 3)

Here \( n_1, n_2, K_{d1}, \) and \( K_{d2} \) are the binding cooperativities and equilibrium dissociation constants of TRmBL2 protein at the two binding regimes, respectively; \( \beta \) is the coefficient determining the relative contribution of the two binding regimes into the net DNA occupancy fraction.

**Free Energy Calculations**—To obtain a zero order estimation of the maximum difference between TRmBL2/HTKb affinities to the most and least probable nucleotide pentamers found in their DNA binding sites, we used Boltzmann statistics approximation. From statistical mechanics, it is known that the probability, \( p_i \), to find protein in the \( i \)th state in thermodynamic equilibrium is

\[
p_i = \frac{N_i e^{-k_BT E_i}}{Z}
\]

(Eq. 4)

Here \( N_i \) is the degeneracy factor of the \( i \)th state, \( E_i \) is the energy of the \( i \)th state, and \( Z = \sum_i (N_i \times \exp(-E_i/k_BT)) \) is the partition function. In this study, the states were indexed according to pentamer type (1024 pentamer sequences in total), in which case the degeneracy factor, \( N_i \), simply equals to the number of occurrences of the \( i \)th pentamer in the cell genome.

Using Equation 4, it is easy to find that the maximum difference between TRmBL2/HTKb affinities to the most and least probable pentamers found in their DNA binding sites, \( \Delta E \), is

\[
\Delta E = k_BT \left( \max_i \left( \frac{p_i}{p_{\text{genome}}} \right) - \min_i \left( \frac{p_i}{p_{\text{genome}}} \right) \right)
\]

(Eq. 5)

Here \( p_{\text{genome}} \) and \( p_{\text{protein}} \) are the occurrence frequencies of the \( i \)th pentamer type in T. kodakarenensis genome and in the DNA-binding sites of TRmBL2/HTKb proteins, respectively; \( \max \) and \( \min \) are the maximum and minimum values of the expression inside the parentheses over all pentamer types.

From Equation 5 it is easy to see that the maximum binding energy difference, \( \Delta E \), is simply the widths of the respective barcodes shown on Fig. 6.

It should be noted that the above estimations can be applied to nucleotide sequences of any size as long as their occurrence frequencies in the cell genome are sufficiently large for reliable statistical analysis. In the case of T. kodakarenensis cells, nucleotide pentamers are the largest oligomers that satisfy this criterion. Indeed, the occurrence number of each pentamer in the genomic DNA is \( >300 \), whereas the occurrence numbers of some hexamers are \( <10 \), which may lead to an error in the evaluation of the protein affinity to such low copy number sequences.

**Results**

**TRmBL2 Cooperatively Polymerizes along DNA Forming Rigid Nucleoprotein Filamentous Patches over a Wide Temperature Range**—Previous studies show that TRmBL2 proteins create extended filamentous complexes along DNA at 50 °C (20). Because T. kodakarenensis can thrive at high temperature (60–100 °C) and survive at temperatures as low as 30 °C, we tested whether formation of these structures is affected by changes in temperature by performing AFM imaging of samples prepared at 25 and 70 °C. We found that at both temperatures, DNAs were covered by extended TRmBL2 patches (Fig. 1D). Centerline tracing of AFM images of naked and TRmBL2-covered DNAs shows that the contour length of DNA does not change much upon TRmBL2 binding to it (naked DNA: 349 ± 3 nm, \( n = 46 \); TRmBL2-covered DNA: 332 ± 4 nm, \( n = 30 \) at 25 °C and 333 ± 3 nm, \( n = 34 \) at 70 °C). On the other hand, its relative extension (i.e. end to end distance normalized to the contour length) increases from 0.40 ± 0.02 (naked DNA) to 0.51 ± 0.03 and 0.48 ± 0.03 (TRmBL2-covered DNA at 25 and 70 °C, respectively). These results indicate that TRmBL2 is a thermoresilient architectural protein that neither wraps nor bridges DNA but instead forms DNA stiffening nucleoprotein filamentous patches in a wide range of temperatures including those optimal for growth of T. kodakarenensis cells.

Interestingly, from Fig. 1D it can be seen that TRmBL2 interacts with DNA quite heterogeneously at both low and high temperatures, which may suggest the possible existence of TRmBL2 binding sequence preference. This hypothesis is further supported by AFM images of TRmBL2-DNA nucleoprotein complexes made at a low protein concentration (6 nM). From Fig. 1B it can be seen that at these conditions only small localized TRmBL2 patches can be seen on DNAs at very similar positions relative to the DNA ends at both 25 and 70 °C, indicating possible existence of TRmBL2 high affinity binding sites. Increase of the protein concentration to 20 nM causes formation of elongated nucleoprotein filaments around these sites on DNAs (see Fig. 1C), which suggests that TRmBL2 binds to DNA with positive cooperativity. Thus, AFM experiments show that TRmBL2 high affinity binding sites on DNA may function as nucleation seeds for the nucleoprotein filaments formation, which likely proceeds with positive cooperativity in a non-sequence-specific manner on the flanking DNA segments. In other words, such high affinity sequences may dictate locations for the TRmBL2 nucleoprotein patches formation.

To determine the DNA binding properties of TRmBL2, we performed a series of single-DNA manipulation experiments using transverse magnetic tweezers as described in our earlier publications (22, 23). By stretching a DNA (48,502 bp) and measuring its force extension curve in the presence of different concentrations of TRmBL2 protein in solution, it is possible to
obtain detailed information about the TrmBL2-DNA interaction and physical characteristics of the resulting nucleoprotein filaments. Our experimental data show that binding of TrmBL2 to DNA results in a larger DNA extension at low, but not at high tension. At 0.1 pN the DNA extension increases by ~30%, whereas at 10 pN the extension change is negligible (Fig. 2A), indicating that TrmBL2 binding mainly causes net DNA stiffening without leading to other DNA distortions such as DNA bending, intercalation, or unwinding (32), in good agreement with the results obtained from the AFM image analysis. Interestingly, the stiffening effect starts to appear in the nanomolar range of TrmBL2 concentrations (Fig. 2A), suggesting a high binding affinity of TrmBL2 to DNA.

At each TrmBL2 concentration, the force extension curve of TrmBL2-DNA nucleoprotein complex fits well to the Marko-Siggia’s formula derived from the worm-like chain polymer model (30) (Fig. 2A). The fitted effective persistence length of TrmBL2-covered DNA rapidly increases with TrmBL2 concentration from 49 ± 2 nm (naked DNA) to 96 ± 8 nm for 60 nM of TrmBL2. Then it plateaus at 96 nm until TrmBL2 amount in solution reaches >150 nM. Further increase of the TrmBL2 concentration above 150 nM leads to the second jump in the persistence length from 96 ± 8 to 122 ± 5 nm, remaining at this value even when the TrmBL2 concentration is as high as 3,000 nM (Fig. 2B). These results suggest that TrmBL2 may have two DNA binding modes that lead to different levels of DNA stiffening in a concentration-dependent manner. This hypothesis is further supported by the presence of high and low affinity binding modes of TrmBL2 to DNA observed in mobility shift assay (data not shown).

To estimate the equilibrium dissociation constant of DNA-TrmBL2 interaction, we calculated the fraction of DNA-binding sites occupied by TrmBL2 at different concentrations of the protein (Equation 1 under “Materials and Methods”). The resulting occupancy fraction shows two-step binding regimes of TrmBL2 to DNA (Fig. 2C), again indicating that TrmBL2 interaction with DNA may be more complex than a single-binding mode picture. Because AFM images of nucleoprotein

FIGURE 1. AFM images of DNA in the absence (A) and presence of 6, 20, and 60 nM TrmBL2 in solution (B, C, and D, respectively). Small patches at 6 nM and elongated nucleoprotein filaments at 20 nM of TrmBL2 indicated by the arrows can be seen on some of DNAs at similar positions relative to the DNA ends, suggesting the existence of TrmBL2 high affinity binding sites.

FIGURE 2. DNA binding properties of TrmBL2. A, force extension curves of λ-DNA at different concentrations of TrmBL2 in solution reveal DNA-stiffening effect caused by formation of TrmBL2 nucleoprotein complexes. Solid lines show the data fitting to the worm-like chain polymer model. B, persistence length of TrmBL2-covered λ-DNA estimated by the experimental data fitting to worm-like chain model as a function of TrmBL2 concentration in solution. C, λ-DNA occupancy fraction by TrmBL2 as a function of the protein concentration. The occupancy fraction was calculated from the data points shown in B using Equation 1. The blue dashed curve shows the data fitting to the Hill equation ($\chi^2 = 0.93$), whereas the red solid curve shows the data fitting to double Hill equation ($\chi^2 = 0.05$). In A, the relative extension of λ-DNA is obtained by normalizing its end to end distance to the contour length (~16.5 μm); each data point is the average of ≥4 experiments. Error bars in all of the panels indicate standard errors.
complexes suggest that TrmBL2 likely forms filaments on DNA with positive cooperativity, the occupancy fraction data points presented on Fig. 2C were fitted to Hill equation (Equation 2), which, however, did not show a good agreement with the experimental data (blue dashed curve). Instead, the double Hill equation (Equation 3) showed much better results (red solid curve). The evaluated Hill coefficients found in the double Hill fitting are $n_1 = 2.5 \pm 0.7$ and $n_2 = 4.0 \pm 1.0$, in good agreement with AFM observations suggesting positive binding cooperativity of TrmBL2 to DNA. The fitted values of the equilibrium dissociation constants are $K_{d1} = 4.5 \pm 0.4$ nM and $K_{d2} = 290 \pm 20$ nM. These results together with AFM images showing TrmBL2 binding to specific sites on DNA at small concentrations indicate that this protein may have a certain binding sequence preference associated with the smaller dissociation constant and a nonspecific binding mode associated with the larger one.

Interestingly, the occupancy fraction data fitting to single-Hill equation describing cooperative protein binding to DNA or Langmuir-type equation describing noncooperative protein binding result in very similar dissociation equilibrium constants of TrmBL2 in the range of 5–7 nM, which are close to $K_{d1}$ of the higher affinity mode (4.5 ± 0.4 nM) obtained using the double Hill equation. This result suggests that although there may be more than one binding mode of TrmBL2 to DNA, there is a predominant one that distinguishes itself by a much lower dissociation equilibrium constant than the other modes.

TrmBL2 Binding to DNA Is Sensitive to Potassium Concentration over the Physiological Range—The existing experimental studies show that a temperature-dependent regulation of intracellular potassium ion concentration in methanogenic archaeal thermophiles may play an important role in their biochemical thermodaptation (33). Because of many similarities between methanogenic thermophiles and *T. kodakarenensis* cells, it is highly probable that the latter may also have a similar thermoregulation of intracellular potassium concentration. Therefore, we investigated the influence of potassium ions on the stability of TrmBL2 nucleoprotein filaments by measuring force extension curves of DNA-TrmBL2 complexes at different KCl concentrations. We found that increasing the concentration of KCl causes reduction in the level of TrmBL2-induced DNA stiffening. Furthermore, at 600 mM KCl, the DNA force extension curve drops slightly below that of a naked DNA measured at 50 mM KCl (Fig. 3), and after switching buffer to 50 mM KCl without TrmBL2, it becomes indistinguishable from that of a naked DNA. These results indicate that the DNA binding affinity of TrmBL2 decreases with the KCl concentration. Consistently, our AFM images show that TrmBL2 protein covers DNAs considerably less at ≥300 mM KCl than at 50 mM KCl in a wide range of temperatures (compare Figs. 4, A and B, and 1D). Although most DNA regions are free from TrmBL2 proteins at high KCl concentrations (≥300 mM), small localized TrmBL2 nucleoprotein patches still can be seen at similar DNA sites as revealed in AFM imaging experiments performed at a low protein concentration at 50 mM KCl (compare Figs. 4 and 1B). These results further support the AFM observations of high affinity TrmBL2 binding sequences on DNA, which are consistent with the two-step binding behavior of TrmBL2 revealed in single-DNA stretching experiments.

Furthermore, the above findings show that nonspecific interaction of TrmBL2 with the negatively charged phosphate backbone of DNA constitutes a large part of the protein DNA-binding energy, which weakens at increased KCl concentrations because of electrostatic screening. To delineate the relative contribution of cations (K$^+$) and anions (Cl$^-$) to TrmBL2-DNA interaction weakening, we performed similar DNA stretching experiments using potassium glutamate working buffer (34), which showed analogous decreasing DNA-stiffening trend with increasing solution ionic strength (data not shown). These results suggest that K$^+$ ions play the main role in the observed electrostatic screening, although slightly higher DNA exten-

FIGURE 4. AFM images of DNA incubated with 60 nM TrmBL2 in the presence of 300 and 600 mM KCl in buffer solution (A and B, respectively) at 25 and 70 °C. At both salt concentrations, small patches of TrmBL2 indicated by the arrows can be seen on some of DNAs at similar sites as in the case of AFM experiments performed at a low protein concentration at 50 mM KCl (see Fig. 1B).
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FIGURE 5. Influence of TrmBL2 on DNA supercoiling. A, twist extension curves of DNA at 0.3- and 1-pN tensions in the absence and presence of a saturating TrmBL2 protein concentration (600 nM). Arrows indicate onsets of the plectonemes formation (i.e., buckling transition point). B, effect of the TrmBL2 concentration on the twist extension curve of DNA at 0.3 pN. TrmBL2 binding to DNA causes symmetry breaking of the twist extension curve presumably because of the structural transition of a part of the DNA to a torsion-relaxing state (melting or/and Z/L-DNA formation) during unwinding. In A and B, the relative extension of the DNA is obtained by normalizing its end to end distance to the contour length (−1.7 μm); each data point is the average of ≥4 experiments. Error bars show standard errors.

As mentioned in the above KCl experiments, indicate that Cl− ions also partially contribute to it, in good agreement with previously published studies (34).

TrmBL2 Binding to DNA Impedes Formation of Supercoiled DNA Plectonemes during Winding and Promotes DNA Structural Transition during Unwinding—All archaea have a circular chromosomal DNA, which is believed to be in a negative supercoiled state for many of these cells. Indeed, archaeal circular plasmids extracted from mesophilic euryarchaeon cells frequently exist in a negative supercoiled plectonemic state (35, 36), which is likely driven by left-handed helical DNA wrapping by archaeal histones at physiological conditions (37, 38). However, circular plasmids isolated from many thermophilic euryarchaeons (including Thermococccaeae family) have a relaxed or even positive supercoiled state, suggesting that some mechanisms exist to counter the histone-induced negative supercoiling. For instance, it is possible that other architectural proteins and/or DNA topoisomerases (35) may play an important role in the chromosomal DNA supercoiling state regulation in these cells.

To date, three families of abundant architectural proteins have been identified in T. kodakarensis cells (20): 1) histones A/B, which wrap DNA; 2) Alba proteins, which form extended nucleoprotein filaments and mediate DNA bridging; and 3) TrmBL2, which forms stiff nucleoprotein filaments but lacks DNA bridging function. Both DNA wrapping by histones and bridging by Alba proteins stabilize the supercoiling state of DNA, whereas the role of TrmBL2 in its regulation is not fully understood.

To investigate the effects of TrmBL2 binding on the DNA supercoiling state, we used a magnetic tweezers setup in which torsionally constrained DNA tethers can be twisted in a controllable and reversible fashion (25). Experimental and theoretical studies show that DNA winding/unwinding causes accumulation of its twist elastic energy, which after reaching a certain threshold either relaxes through the DNA chiral bending and plectonemes formation or via transition of a part of the DNA into torsion-relaxing structural states (25, 39–44). The latter include formation of overwound right-handed P-DNA during DNA winding, and a combination of DNA melting and/or transition into a left-handed Z/L-form during DNA unwinding.

By twisting a 5-kilobase pair DNA at a low tension (0.3 pN), we found in our experiments that both DNA winding and unwinding cause DNA folding caused by plectoneme formation, leading to a symmetric twist extension curve (Fig. 5, A and B, black data points). The onset of the DNA plectonem supercoiling, often referred to as the buckling transition, marks a point after which the DNA extension linearly decreases with further DNA winding/unwinding (see arrows in Fig. 5A). At this point, superhelical density (also often referred to as linking number density) of the DNA, σ, reaches a threshold value at which initial chiral bending of the DNA takes place.

At a higher DNA tension (1 pN), a larger energy input is required to initiate DNA chiral bending. As a result, transition of a part of the DNA to an alternative structural state (DNA melting and/or Z-DNA formation) becomes a more energetically favorable mode of the DNA twist relaxation during its unwinding (39–42, 44), leading to the symmetry breaking of the twist extension curve (Fig. 5A, blue data points), whereas during winding, DNA still relaxes via formation of supercoiled plectonemes, although, the higher energy price of the DNA bending causes a shift of the buckling transition to a larger superhelical density.

Our experiments show that addition of a saturating concentration of TrmBL2 (600 nM) to DNA increases the superhelical density of the buckling transition during DNA winding by a factor of ~2 at both 0.3- and 1-pN tensions (arrows in Fig. 5A). Moreover, TrmBL2 protein binding to the DNA causes the symmetry breaking of its twist extension curve even at low tension (0.3 pN), in contrast to the symmetric twist extension curve of a naked DNA at the same force. This asymmetric behavior of the DNA twist extension curve in the presence of TrmBL2 is reminiscent of that observed for a naked DNA at the higher tension of 1pN. These results suggest that TrmBL2 binding to DNA strongly affects its physical state by impeding the DNA buckling transition during winding and promoting structural transformation of a part of the DNA during unwinding. Interestingly, these effects start to appear at very low concentrations of TrmBL2 (~10 nM; see Fig. 5B),
which is consistent with the low equilibrium dissociation constant (~5 nM) of TrmBL2 measured in our DNA stretching experiments.

Both the increased buckling transition threshold of TrmBL2-covered DNA during its winding and the promoted switching to torsion-relaxing structures during its unwinding can be understood based on the observed DNA-stiffening effect by TrmBL2 proteins. Indeed, rigid TrmBL2 nucleoprotein filaments hinder DNA bending and plectonemes formation. Therefore, accumulation of a larger amount of the DNA twist elastic energy is required to initiate the buckling transition during DNA winding. On the other hand, during DNA unwinding, the same TrmBL2-induced stiffening effect makes relaxation of the accumulated twist energy via formation of plectonemes less energetically favorable than via DNA structural transition pathway, resulting in asymmetric twist extension curve of the DNA. Because our AFM images suggest that TrmBL2 creates rigid nucleoprotein filamentous patches on DNA at physiologically relevant temperatures (70 °C), as well as at room temperature (Fig. 1D), it is probable that the TrmBL2-induced effects described above may also take place in vivo conditions.

**TrmBL2 and Archaeal Histones Preferentially Bind to G/C-rich Parts of the Genomic DNA in Vivo—**To determine the possible sequence preference of TrmBL2 proteins, we reanalyzed the sequencing data that revealed positions of TrmBL2 binding sites on the genomic DNA protected against MNase I digestion (20). Because TrmBL2 is a recently identified protein, its exact DNA binding site size still remains unknown. Therefore, we estimated the difference in TrmBL2 binding affinities to short pentamer nucleotide sequences instead. For this purpose, we calculated the frequencies of all possible 1024 nucleotide pentamers in the whole cell genome relative to their occurrences in binding sites of TrmBL2 and plotted them in the form of a color barcode shown on Fig. 6 (see “Materials and Methods” for more details). Similar analysis was also performed for *T. kodakarensis* histones as a control.

From Fig. 6, it can be seen that both TrmBL2 and histones have binding preference to G/C-rich sequences, whereas TrmBL2 proteins, but not histones, also have some affinity for A/T-rich sequences. Identical tetra- and hexamer nucleotide sequences analysis also leads to very similar conclusions, indicating robustness of these results (data not shown). Observed sequence selectivities of TrmBL2 and histones are consistent with previous experimental studies showing that archaeal nucleosomes tend to bind to G/C-rich genome regions and avoid A/T-rich transcription initiation and termination sequences (45) and that TrmBL2 protein functions as a global expression regulator of many *T. kodakarensis* genes by binding to some A/T-rich promoter regions (20).

Additionally, our estimations show that the maximum difference between *T. kodakarensis* histone affinities to the most and least probable pentamers found in their DNA binding sites is ~1.6 *k*T (see “Materials and Methods”). It has been reported that *T. kodakarensis* histones form multimeric structures primarily in the form of tetramers binding on average to 60 bp of DNA (16, 45), which is 12 times larger than a nucleotide pentamer. Therefore, 1.6 *k*T energy difference per a nucleotide pentamer results in a large binding energy gap of ~19.2 *k*T between the most and least probable histone positions on the genomic DNA. This result explains the high sequence selectivity of archaeal nucleosome positioning observed both in vivo and in vitro (45). Similar analysis performed for TrmBL2 reveals that on average this protein is not as selective for DNA positioning as *T. kodakarensis* histones. The calculated difference between the TrmBL2 affinities to the most and least probable pentamer sequences (~1.0 *k*T) is comparable with that of histones. However, because of a much smaller binding site size of ~10–15 bp typical for a helix-turn-helix protein motif (46), the resulting binding energy difference between the most and least probable positions of a TrmBL2 protein on the genomic DNA in vivo is only ~2–3 *k*T, which is consistent with the two equilibrium dissociation constants of TrmBL2 measured in our in vitro assays (4.5 and 290 nM). Thus, TrmBL2 proteins on average have rather small sequence selectivity, which is in sharp contrast to the localization of TrmBL2 on promoter and coding regions of specific genes in vivo (20), raising a question regarding molecular mechanisms responsible for TrmBL2 precise positioning on the chromosomal DNA (see “Discussion”).

**T. kodakarensis TrmBL2 and HTkB Mutually Occlude Each Other for DNA Binding—**Because both TrmBL2 and histones have overlapping sequence preferences in vivo, they may compete with each other for DNA binding inside living cells. We hypothesize that such a competition could lead to a highly dynamic structure of the *T. kodakarensis* chromatin because these two proteins have opposite effects on DNA organization. Archaeal histones wrap DNA, forming highly compact structures similar to eukaryotic nucleosomes, whereas TrmBL2 proteins form rigid filamentous nucleoprotein complexes along DNA, which prevent the DNA folding into a coiled configuration.
AFM images show that both HTkB and TrmBL2 form thermostable structures with DNA (Figs. 7, A–B, and 1, B–D, respectively). Thus, the proposed hypothesis about DNA binding competition between these two proteins can be tested at a single-DNA level by the magnetic tweezers setup described above. For this purpose, the extension of a-DNA held at force of 1 pN was measured during subsequent addition of HTkB and TrmBL2 proteins to it. The left panel in Fig. 7C (50 mM KCl) shows a typical DNA extension change for the following four sequential stages: naked DNA, +TrmBL2 (addition of TrmBL2 to solution), −TrmBL2 (removal of free TrmBL2 from solution), and +HTkB (addition of HTkB). We found that addition of TrmBL2 to the DNA (+TrmBL2) leads to a slight increase in the DNA extension caused by the nucleoprotein stiffening effect. Moreover, removal of free TrmBL2 from solution at the next stage (−TrmBL2) does not affect the DNA extension, indicating that TrmBL2, once forming a filament on DNA, has a very low dissociation rate in the absence of free TrmBL2 in solution. Subsequent addition of HTkB (+HTkB) causes only a moderate DNA folding at t = 90 min, which could be in part explained by HTkB binding to DNA regions free from TrmBL2 proteins. Further, progressive DNA extension decrease with the rate dependent on the HTkB concentration (the higher the concentration, the faster the DNA fold-

FIGURE 7. DNA binding competition between TrmBL2 and HTkB. A and B, AFM images of DNA in the presence of 0.6 nM HTkB at 25 °C (A) and 70 °C (B) show that archaeal histones induce DNA compaction equally well at low and high temperatures. C and D, single λ-DNA studies of HTkB and TrmBL2 binding competition at low (C) and high (D) KCl concentrations. The relative extension of a mechanically stretched λ-DNA at 1 pN was monitored as a function of time. TrmBL2 and HTkB were added to/removed from solution in different orders to investigate how one protein displaces the other from the DNA.
ing is) suggests that HTkB may facilitate dissociation of DNA-prebound TrmBL2.

By adding HTkB and TrmBL2 proteins to the DNA in the reverse order (naked DNA, +HTkB, −HTkB, and +TrmBL2), a very different DNA extension change pattern can be revealed (Fig. 7C, right panel). Introduction of histones B into solution (+HTkB) causes a rapid DNA extension decrease, over a much larger length scale than that observed in the case when DNA is preincubated with TrmBL2 (Fig. 7C, compare left and right panels). Upon removal of free HTkB from solution (−HTkB), the DNA extension increases slightly, indicating dissociation of a number of histones from the DNA and suggesting that they interact with some DNA regions in a dynamic manner. Finally, addition of TrmBL2 (+TrmBL2) causes a slow progressive increase of the DNA extension caused by the displacement of histones by TrmBL2. However, this time it more strongly depends on the fraction of DNA preoccupied by HTkB than the concentration of TrmBL2 in solution, suggesting that the intrinsic dissociation rate of DNA-bound HTkB is likely the major rate-limiting step of this process.

Similar experiments performed at a higher KCl concentration of 300 mM show that the histone affinity to DNA strongly decreases with increasing ionic strength of solution. As a result, DNA-bound HTkB becomes more easily displaced from DNA by TrmBL2 proteins upon their addition into solution (Fig. 7D).

Taken together, our results indicate that TrmBL2 and HTkB mutually occlude each other for DNA binding at a low ionic strength, whereas at a high ionic strength, TrmBL2 becomes more competitive in DNA binding compared with HTkB. Therefore, should such competition for DNA binding sites between TrmBL2 and archael histones occur in vivo, our data suggest that it would be regulated by the cytoplasmic potassium ion concentration.

**Influence of DNA Supercoiling on DNA Binding Competition of TrmBL2 and HTkB**—As we have shown in Fig. 5, twisting of TrmBL2-DNA nucleoprotein complexes has a profound effect on the physical state of DNA. Thus, it is likely that DNA supercoiling might strongly modulate the observed DNA binding competition between TrmBL2 and HTkB proteins.

To investigate this question, we first measured the effect of HTkB proteins on DNA supercoiling in 50 mM KCl solution. Fig. 8 shows that even the addition of a low concentration of HTkB (0.6 nM) leads to significant changes in the twist extension curve of DNA at a low tension (0.3 pN) compared with that of a naked DNA (red and black curves, respectively). The maximum DNA extension reduces by ~7-fold in presence of HTkB, presumably as a result of the DNA wrapping by archael histones. Furthermore, the profile of the twist extension curve becomes more flat, and the center peak slightly shifts to the positive side (σ = −0.01), indicating that HTkB has a slight preference to wrap DNA in a right-handed manner, which is in good agreement with previously published bulk experiments performed at the similar salt concentration (37). Despite this weak DNA wrapping preference, the overall broadening of the twist extension curve suggests that HTkB-DNA complexes can rather easily flip between right- and left-handed configurations in response to DNA winding/unwinding. This observation is supported by existing experimental studies showing that similar archael histones handeness flipping can be induced by change in the solution ionic strength (37, 38). Interestingly, structurally homologous eukaryotic H3-H4 histone tetramers (15) also can switch their helical handedness in response to DNA supercoiling (47–49), indicating universality of this phenomenon among histone-like proteins from different organisms.

Very distinct effects of TrmBL2 and HTkB on the profile of the twist extension curve make it possible to delineate their individual behavior in the case when DNA is incubated with a mixture of these proteins. From Fig. 8, it can be seen that the DNA binding competition between TrmBL2 and HTkB is strongly affected by the level of DNA supercoiling. Indeed, at low and high DNA superhelical densities (σ < −0.03 and σ > 0.05), the resulting twist extension curve corresponding to the proteins mixture (green) approaches that of HTkB-covered DNA (red), indicating that under such conditions TrmBL2, but not HTkB, interaction with DNA is likely reduced, whereas in the middle range (−0.03 < σ < 0.03), both proteins are present on the DNA. On the plot, each data point is the average of ≥3 experiments. Error bars show standard errors.

![FIGURE 8. Influence of DNA supercoiling on TrmBL2 and HTkB binding competition. Comparison of the DNA twist extension curve measured in the presence of TrmBL2 and HTkB mixture (green) to those of HTkB-covered (red) and TrmBL2-covered (cyan) DNAs shows that at a high positive/negative superhelical densities (σ < −0.03 and σ > 0.05) DNA binding by TrmBL2, but not HTkB, is reduced. On the other hand, in the middle range (−0.03 < σ < 0.03), both proteins are present on the DNA. On the plot, each data point is the average of ≥3 experiments. Error bars show standard errors.](image-url)
ods”). It was found that this protein is maintained in living cells at a sufficiently high level of $C_{\text{protein total}} = 37 \pm 7 \mu M$. Although the total intracellular concentration of TrmBL2 is high, it should be noted that most of the protein is likely to be bound to the large chromosomal DNA ($\sim 2.1$ Mbp), so the concentration of free/DNA-unbound TrmBL2 should be considerably lower than $37 \mu M$.

Indeed, it is a very well known fact of chemical kinetics theory that in the case when the concentration of available protein binding sites, $C_{\text{binding sites}}$, is larger than the protein concentration ($C_{\text{protein total}} = 85 \pm 15 \mu M$), the protein concentration of free/unbound protein will be of the order of $K_d$. Simple calculations based on the average cell diameter ($\sim 1.5 \mu m$, (50)) and the typical size of helix-turn-helix protein consensus sequence ($\sim 10-15$bp, (46)) show that this is the case for TrmBL2 protein because $C_{\text{binding sites}}$ is larger than the protein concentration $C_{\text{protein total}} = 37 \pm 7 \mu M$.

The presence of other architectural proteins (like HTkB) that compete with TrmBL2 for binding sites on the chromosomal DNA slightly changes the above picture. Indeed, these proteins reduce the amount of DNA-bound TrmBL2, thus increasing the intracellular concentration of free TrmBL2. Therefore, the $in vivo$ concentration of TrmBL2 may range from several $nM$ up to a few $\mu M$ depending on competition with other DNA-binding proteins, which is the concentration range tested in our experiments.

**Discussion**

To maintain a highly organized tight packaging of the large chromosomal DNA ($\sim 2.1$ Mbp) in a small cellular volume of only $\sim 1-4$ $\mu m^3$, *T. kodakarensis* cells use several different types of DNA-architectural proteins. So far, three families of highly abundant DNA-architectural proteins have been identified in these cells: 1) archaeal histones A/B, 2) Alba, and 3) TrmBL2 (20). Previously, it was shown that archaeal histones wrap DNA and form nucleosome-like particles (14–16), whereas Alba proteins mediate DNA bridging (17, 18). Thus, both of these proteins can contribute to the genomic DNA packaging: histone, via the direct DNA condensation, and Alba, via DNA loops formation and stabilization of plecomic DNA supercoils. Here we show that, in contrast to histones and Alba proteins, TrmBL2 antagonizes packaging of DNA by forming stiff nucleoprotein filamentous structures. It was found that these filament can modulate the physical organization of DNA by competing with histones for DNA binding in a salt- and DNA supercoiling-dependent manner. Thus, TrmBL2 likely acts as a regulator of the chromosome condensation level in *T. kodakarensis* cells, a function that may further impact the global gene transcription profile.

**Sequence Selectivity of TrmBL2**—Analysis of TrmBL2 binding sequences suggests that the average sequence selectivity of this DNA-architectural protein is rather low. This lack of specificity is in stark contrast with the role of TrmBL2 as a global repressor of many specific *T. kodakarensis* genes, a function
plasmic potassium ion concentration. It also cannot be ruled out that other DNA-architectural proteins may be involved in a similar DNA binding competition with TrmBL2.

**Impact of DNA Supercoiling on TrmBL2 and Archaeal Histones Competitive Binding**—Our experimental data indicate that in addition to intracellular potassium ion concentration, the DNA binding competition between TrmBL2 and archael histones may also be modulated by the level of DNA supercoiling (Fig. 8), which can be fully understood based on the distinct responses of these proteins to DNA winding/unwinding. Indeed, as was shown in this study, TrmBL2 nucleoprotein complexes impede formation of supercoiled plectonemes during DNA twisting by delaying its buckling transition, which suggests that the chiral bending of DNA taking place during this process is disfavored by TrmBL2. On the other hand, both positive and negative supercoiling of DNA likely increases the stability of HTkB-DNA complexes because archael histones can wrap DNA in both left- and right-handed manners. As a result of these mechanical effects, HTkB binding to DNA may become more energetically favored over that of TrmBL2 at high positive/negative DNA torsions. This might explain the observed decrease in TrmBL2 binding to DNA in the presence of HTkB at low (\(\sigma < -0.03\)) and high (\(\sigma > 0.05\)) superhelical densities and disappearance of the TrmBL2-induced structural transition of DNA at negative superhelical densities (Fig. 8). On the other hand, at low levels of DNA twisting (\(-0.03 < \sigma < 0.03\)), the effect of DNA supercoiling on the binding properties of TrmBL2 and HTkB becomes small, and the balance between these two proteins shifts in favor of TrmBL2. As for the narrow range of \(0.03 \leq \sigma \leq 0.05\), our experimental data show that the twist extension curve of DNA measured in the presence of the proteins mixture at these superhelical densities goes higher than the respective curves of HTkB- or TrmBL2-covered DNAs, indicating a partial relaxation of positively supercoiled DNA plectonemes caused by the proteins cooperative behavior. Indeed, HTkB binding to DNA causes formation of the right-handed nucleoprotein complexes, which adsorb a portion of the accumulated positive DNA supercoils. This leads to a partial torsion relaxation in the rest of the DNA on which TrmBL2 proteins may polymerize into stiff nucleoprotein filamentous patches impeding DNA plectoneme development.

The above results demonstrate that the DNA occupancy by TrmBL2 and HTkB proteins can be strongly modulated via changes in the DNA supercoiling level, suggesting that topoisomerase activity likely plays an important role in controlling the balance between these two proteins in the chromosomal DNA organization in vivo. Interestingly, previous experimental studies show that the chromosomal DNA in bacterial cells is arranged in a form of nucleoid, in which it is divided in multiple topologically isolated domains by DNA-bridging proteins (8, 12, 56). Although the higher order organization of archael genome has not been explicitly studied to date, the abundance of DNA-bridging proteins (like Alba) in archael cells indicates that it may also have a looped domains structure similar to that found in bacteria (8). In this case, the balance between the archael histones and TrmBL2 in such topologically isolated genome regions may be independently regulated by topo-

isomerase activity, resulting in differential positioning of these two proteins along the chromosomal DNA.

It should be noted that the supercoiling-dependent modulation of the DNA binding competition between TrmBL2 and HTkB revealed in this study is a natural result of their distinct mechanical responses to DNA twisting. Therefore, the above conclusions have wide biological implications because they can be further generalized to understand collective behavior of DNA-stiffening and DNA-wrapping/bending proteins in other cell types, especially taking into account that an increasing number of architectural proteins that can form rigid nucleo-protein filaments have been recently identified as crucial DNA organizing elements and gene transcription factors in different bacteria (31, 51, 52, 57–61).

**Role of TrmBL2 DNA Binding Properties in Its Possible Regulatory Function in Vivo**—In our previous study, we showed that TrmBL2 binding to the promoter regions of the genes leads to their repression (possibly by blocking transcription factor/RNAP access to promoters), whereas formation of TrmBL2 nucleoprotein complexes on the coding sequences of the genes has a negligible influence on the transcription level (20). In addition, our new experimental data indicate that TrmBL2 interaction with DNA is sensitive to the surrounding potassium ion concentration, with stronger protein binding observed at lower KCl concentrations. Together, these results suggest that TrmBL2 may play an important role in the global gene transcription regulation in *T. kodakarensis* cells in response to environmental changes. Indeed, it was previously found that the cytoplasmic potassium ion concentration in methanogen archaea hyperthermophiles is maintained at a high level at optimal growth temperature conditions, whereas temperature decrease below optimal leads to a considerable drop in the K$^+$ ion concentration in the cytoplasm (33). Although to date there are no such data for *T. kodakarensis* cells, it is likely that a similar thermoregulation of K$^+$ ion concentration also takes place inside *T. kodakarensis* cells because of many similarities between these two thermophilic archaea cells. Thus, environmental temperature decrease and the subsequent drop in the cytoplasmic K$^+$ ion concentration in *T. kodakarensis* cells may lead to increased polymerization of TrmBL2 filaments on segments flanking high affinity binding sites on the chromosomal DNA. Potentially, such elongated nucleoprotein filaments may reach some of nearby promoter sequences, causing silencing of the respective genes.

Previously, it was demonstrated that TrmBL2 extensively covers coding sequences of operons encoding RNAP A'/A"/B/H and 16S/23S rRNAs subunits in vivo at an optimal growth temperature of 85 °C. However, these TrmBL2 nucleoprotein filaments do not have a strong influence on the transcription levels of the above genes compared with the \(\DeltaTrmBL2\) mutant (20), possibly because RNAPs can easily displace TrmBL2 proteins from DNA during transcription elongation. Although the physiological role of these filaments is unclear, based on the regulatory mechanism proposed here, we hypothesize that at lower temperatures, because of the intracellular K$^+$ ion concentration drop, TrmBL2 filaments bound to the coding region of RNAP and rRNA genes may extend into the promoter regions, blocking their access to transcription proteins. In
TrmBL2-DNA Interaction and Competition with Histones

This will lead to RNAP and rRNA genes repression, which may be important for T. kodakarensis cells survival at low temperatures.

Indeed, at optimal growth conditions, cells require many ribosomes to synthesize new proteins. Thus, the majority of RNAPs primarily produce new rRNA subunits (62, 63). However, in deteriorating environmental conditions, cells often slow down the ribosomes’ production rate (62, 63) and thus decrease the global level of protein synthesis, switching most of the available resources to survival molecular systems. Therefore, the proposed temperature-controlled TrmBL2 regulatory mechanism of gene silencing may be a part of such a cell preserving system in hyperthermophilic T. kodakarensis cells, allowing them to survive at low temperatures. This prediction may be further tested by measuring RNAP A'/A'/B/H and 16S/23S rRNAs subunits transcription level at unfavorably low temperature growth conditions in wild type and ΔTrmBL2 mutant cells in future studies.

Maintenance of DNA Stability at High Temperatures in T. kodakarensis Cells—As extreme thermophiles, T. kodakarensis cells thrive at temperatures of ~60–100 °C (50), which are high enough to cause destabilization of the chromosomal DNA structure. Moreover, at highly transcribed regions of the genome (such as 16S/23S rRNAs genes), the DNA stability may be further compromised by RNAP-generated upstream negative torque (64), which in the presence of TrmBL2 nucleoprotein filaments can potentially induce the DNA transition into torsion-relaxing structures including melted DNA. These observations raise a question regarding molecular mechanisms responsible for maintenance of the chromosomal DNA integrity at such extreme conditions.

To protect the chromosomal DNA against melting, hyperthermophilic archaea cells evolved several molecular mechanisms that counter the DNA destabilizing factors mentioned above. As revealed in previous studies, hyperthermophilic archaeal cells use both high intracellular potassium ions concentration (33) and reverse DNA gyrases generating positive torsional stress in the chromosomal DNA (35, 36) to maintain its stability at high temperatures. Our results suggest that in addition to these two key mechanisms, highly transcribed regions of the T. kodakarensis genome may be further stabilized by supercoiling-induced displacement of a part of DNA-bound TrmBL2 proteins by archaeal histones, resulting in suppression of the TrmBL2-induced DNA structural transition and possible melting at negative torques (Fig. 8). Moreover, it cannot be ruled out that the archaeal histones switching to a left-handed DNA wrapping configuration at negative torques may also contribute to the chromosomal DNA stabilization via adsorption of the RNAP-generated upstream negative supercoils leading to torsional relaxation of the rest of DNA. Interestingly, previous experimental studies suggest that thermophilic bacteria for a similar purpose may use DNA-bending architectural proteins such as HU that can relax negative torsional stress in DNA by promoting plectonemes formation (65). Overall, our results indicate that in addition to contributing to TrmBL2 positioning along the genome, supercoiling-dependent modulation of DNA binding competition between archaeal histones and TrmBL2 proteins may have another possible physiological role: maintenance of the chromosomal DNA stability at high temperatures.

Acknowledgments—We thank Nozomi Takeda and the Instrumental Analysis Division, Equipment Management Center, Creative Research Institution, Hokkaido University (Japan) for the amino acid analysis of HTKB. Also we thank Peiwen Cong (Mechanobiology Institute at National University of Singapore) for help in AFM images analysis.

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