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RNA Polymerase: Chromosome Domain Boundary Maker and Regulator of Supercoil Density

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Summary

Most bacterial chromosomes and plasmids are covalently closed circular molecules that are maintained in a dynamic supercoiled state. Average supercoil density differs significantly between *E. coli* and *Salmonella*. Two related questions are: 1) What protein(s) create supercoil domain boundaries in a bacterial chromosome? 2) How is supercoil density regulated in different bacterial species? RNA polymerase plays pivotal roles in both of these topological phenomena.

Supercoil Structure of Bacterial Chromosomes

Supercoil density is a function (σ) that is represented by a ratio of the double helix linking difference (Lk) of covalently closed circular DNA relative to a nicked or relaxed molecule (Lk_0) of the same sequence (approximately 1 link per 10.6 bp of DNA). Both large chromosomes and small plasmid DNAs in *Salmonella* and *E. coli* are under-wound relative to standard B-form DNA, but WT strains of both species maintain significantly different values of σ (-0.060 and -0.070 respectively) [1]. A variety of techniques demonstrate that σ is composed of two nearly equal components: half of σ is constrained by moderate to abundant nucleoid associated proteins (σ_C) and the other half is unconstrained and freely diffusible (σ_D) so that $\sigma = \sigma_C + \sigma_D$.

Diffusible supercoiling (σ_D) causes DNA to collapse into a tightly interwound plectonemic structure, which represents the first level of DNA condensation and allows a 4 Mb chromosome to fit into the small space available in a bacterial cell. The free energy (G) of supercoiled DNA increases as a square of superhelix density, and the interwound DNA strands move by plectonemic branching and slithering over distances of 10 – 100 KB in vivo [2–4]. This dynamic DNA network promotes protein-protein interactions required for site-specific recombination (FIGURE 1), transposition, initiation of DNA replication, and transcription [5]. For $\gamma\delta$ resolution assays, the efficiency of recombination varies by 100 fold for cells growing within the physiological relevant range of σ_D from 0 to about -0.045 .

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Constrained supercoiling is generated by the binding, bending, and looping of short segments of DNA to the surface of chromosome-associated proteins [6, 7]. In *E. coli* and *Salmonella*, supercoiling is partitioned nearly equally between these two states [8–10]. A family of small abundant proteins that contribute to constrained supercoiling includes IHF, HU, H-NS StpA, and FIS [11–13]. In addition to the small proteins, the chromosome is also shaped by large proteins including the Structural Maintenance of Chromosome (SMC) complex, also called the MukBEF condensin [14], RNA Polymerase (RNAP) [15], transcription factor Rho, and the motor protein FtsK [16].

RNAP Blocks Supercoil Diffusion

RNA transcription has two distinct topological consequences for chromosome structure inside living cells. In operons that are moderately or highly transcribed, RNAP creates a barrier that stops supercoil diffusion across the transcribed region. This effect was first uncovered in experiments designed to find sequence-specific supercoil barriers in *Salmonella*. Deng [17] conducted a chromosome walk in which she isolated transposons carrying supercoil-dependent resolvase recombination sites that were inserted at many positions spanning a 200 kb segment of the *Salmonella* genome. The experiment was designed to combine pairs of insertions in a tester strain and measure the supercoil-dependent deletion efficiency for each pair of sites. One pair of sites showed a dramatic drop in resolution efficiency and this interval appeared to include a natural supercoil boundary. Further analysis revealed that the barrier was actually caused by a PCR induced mutation in one of the transposons carrying a $\gamma\delta$ Res site. The mutation inactivated the Tet repressor and caused unregulated transcription of *tetA* gene. It was *tetA* transcription that blocked supercoil diffusion. To prove this point, Deng made a new element in which a *lacZ* gene was fused to the TetA promoter and a functional TetR repressor. For the first time, Deng proved that transcription of the *lacZ* gene generated a new barrier to supercoil diffusion by inducing expression with anhydro-tetracycline. A barrier appeared within minutes of induction and disappeared within minutes after inducer was washed out of the cells [18]. Subsequent work proved that every site tested in the *Salmonella* genome that was picked from microarray data showing high transcription rates had a supercoil barrier, whether the operon encoded a protein or a non-translated RNA [19].

RNAP: Also a Primary Regulator of Diffusible Supercoil Density

Determining which genes regulate supercoil density has been another long-standing puzzle. The obvious genes known to influence σ_D are topoisomerases. Topo I (also called ω protein) will enzymatically relax (–) supercoils [20], and DNA gyrase [21] is the only topoisomerase that can catalytically increase the (–) supercoiling of relaxed plasmids. With these two facts in mind, Menzel and Gellert [22] proposed the homeostatic control mechanism. In this model, if DNA becomes hyper-supercoiled, the Topo I promoter is activated to recruit RNAP and increase relaxing potential by making more cellular Topo I. When DNA becomes strongly relaxed, *gyrA* and *gyrB* promoters are activated to produce gyrase and increase (–) supercoiling. This mechanism works because promoters of *GyrB* and *TopA* are activated in the appropriate way in many different bacteria. However, experimental manipulation of gyrase and Topo I levels in vivo showed that the abundance of these

proteins alone does not cause dramatic changes in supercoil density. For example, a 10% increase or decrease in gyrase or Topo I only results in a 1.5% change in supercoil density [23]. This means that a large deviation in supercoiling would require a considerable time to re-establish a new steady state equilibrium. It turned out that the supercoil regulatory system is more complex than the simple topoisomerase competition model predicts.

Liu and Wang [24] proposed a second mechanism in 1987 (FIGURE 2) that generates both (–) and (+) supercoils over the time-course of transcription, which occurs within seconds to minutes. They argued that the large mass of an RNA polymerase molecule and the RNA message that is attached to translating ribosomes would require the template DNA to rotate along its axis rather than the RNA polymerase rotating around the double helix. The model predicts twin domains of supercoiling with (–) supercoils being generated behind the machinery and a loss of (–) supercoils or possibly (+) supercoils accumulating downstream of a transcription terminator.

The twin domain model was tested in *Salmonella* by measuring the supercoil density across the *rrnG* operon and in two 9 kb regions, one located upstream of the *rrnG* promoter and the second downstream of the *rrnG* terminator [19]. These experiments demonstrated that a significant supercoil differential exists in WT cells on opposite sides of a highly transcribed operon, confirming the twin domain model. The upstream region was hyper-supercoiled relative to the chromosomal mean, whereas the downstream sector was depleted of (–) supercoils. The hypothesis that transcription caused this local gradient was confirmed by transiently blocking transcription with the drug rifampicin, which stops transcription initiation but allows elongation and termination to proceed. When transcription was disrupted, supercoiling levels on both flanking regions equilibrated, proving that the movement of RNA polymerase through the transcribed track generates the twin domain behavior predicted by Liu and Wang.

Booker [18] also analyzed twin domain effects in strains carrying mutations in Topo I and TS alleles of gyrase in cultures growing at 30° (FIGURE 2). TS mutants in *gyrA* showed a significant loss of (–) supercoiling in the downstream sector compared to the upstream region. Contrariwise, mutations in TopoI showed the reverse behavior, the upstream region became hyper-negatively supercoiled compared to the downstream region. Thus, the areas where each topoisomerase must work the hardest to neutralize a twin domain effect showed the strongest impact of a mutant enzyme.

RNAP Elongation Rates are Linked to the Gyrase Catalytic Rate

In addition to demonstrating the Liu and Wang twin domain effect in a chromosomal context, experiments with *gyrB*^{TS} mutants showed that RNA transcription can deplete diffusible supercoiling from an entire chromosome. Booker also measured the effect of one important TS mutant, *gyrB652*. The GyrB652 protein has an R436-S substitution in the TOPRIM domain, which undergoes a conformation change during the catalytic cycle. But GyrB652 is not a classic TS enzyme that unfolds or loses activity at 42°. It has a slower catalytic rate than WT at all temperatures from 30 to 42° [25]. Cells stop growing when the medium and temperature create a toxic growth rate that exceeds the ability of gyrase to

maintain supercoiling near the terminus of replication. The *gyrB652^{TS}* mutation caused supercoiling to disappear downstream of *rrnG*. To see if this loss was general or restricted to *rrn* genes, Rovinskiy [26] measured supercoil densities at 30° for 8 different positions in the *Salmonella* chromosome (FIGURE 3). The results showed that the entire chromosome loses nearly all (>95%) diffusible supercoils. Nonetheless, the cultures grew at about half the rate of WT cells. Therefore transcription in a slow gyrase mutant may deplete diffusible supercoils from the entire genome.

To see competing rates of transcription and gyrase supercoiling drives the loss of supercoiling, rifampicin was added to cultures for 15 min and then washed out. Transient transcription disruption allowed cells to recover 80% of the WT supercoiling level, confirming that pressure from RNAP causes chromosome supercoil depletion. Rovinskiy et al. [25] also tested the possibility that supercoil depletion alters the rate of RNAP catalysis. RNA polymerase elongation rates were measured for *lac* operons placed at the 8 chromosome locations. RNAP chain extension rates fell from a mean of 52 nt/sec in WT strains to 32 nt/sec in *gyrB652* strains. With the slow transcription rate, the cell doubling time increased by nearly 2 fold. These results prove that growth rate and gyrase efficiency are linked to the activity of RNA polymerase and the DNA topology predicted by the twin domain model is a dominant force in cell growth. Finally, what happens if one slows down RNA polymerase in cells with a WT complement of topoisomerases? Introduction of an RNAP β' subunit with a 6 amino acid deletion (RpoC 215–220) that mimics the stringent response at all growth conditions caused a 15% increase in supercoil density throughout the chromosome [25]. When WT gyrase gains time to dissipate the (+) domain effects of transcription, the chromosome moves to higher supercoil set point. The rate of transcription elongation and the catalytic turnover rate of gyrase supercoiling are under strong selection to match during optimal growth conditions in WT cells.

General Conclusions

In final analysis, we can think retrospectively about what we should have thought but were unable to see without a chromosome supercoil assay. Knowing that supercoil diffusion proceeds by plectonemic branching/slithering [2], it should have been obvious that highly transcribed genes like the *rrn* operons would block supercoil diffusion. Beautiful images by Sara French [27] show 50–100 RNAP molecular “Christmas trees” (see FIGURE 2) that would clearly block the ability of sites on opposing ends of this operon from adopting the required supercoil tangle for recombination (FIGURE 1) [28]. But our results allow us now to make predictions about growth rate, transcription, and supercoil density in distantly related bacterial species. Many bacteria have slower growth rates and longer doubling times than the 20–25 min values common in *E. coli* and other Gram negative enteric organisms. *Caulobacter crescentus*, *Borrelia burgdorferi*, and *Mycobacterium tuberculosis* (*M. tb*) are examples of organisms with doubling times that range from 5–14 hours. Growth rates depend on the capacity to make new proteins and organisms with slow growth rates have only 1 or 2 ribosomal RNA operons. In such organisms the RNA polymerase extension rates, the catalytic speed of gyrase, and mean supercoil density might all be significantly slower than those observed in fast growing organisms. For example, DNA gyrase from *M. tb* shows a very limited ability to catalyze negative supercoiling but it appears to function more

actively in decatenation of DNA compared to gyrases from faster growing species [29, 30]. Understanding the differences may be important for choosing the best uses of antibacterial chemotherapy. For example, poisoning *M. tb* gyrase for extended times with high doses of Novobiocin or a fluoroquinolone may hinder growth of *M. tb*, but have complicated consequences for beneficial bacteria that detoxify a metabolite or protect a unique microbial niche from association with aggressive microbes that do harm,

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FIGURE 1.

In vitro and *in vivo* $\gamma\delta$ Resolution Reaction Dependence on (–) Supercoiling. **A.** $\gamma\delta$ resolution reactions occur within a pair of 114 bp sites (Res). Three sub-sites bind a dimer of the resolvase; these are labeled I (blue), II (red), and III (yellow.) A productive synapse requires the Res sites to entrap 3 negative crossings of DNA. **B.** Supercoiling promotes dynamic movement to create the three-node synapse via plectonemic branching and slithering. DNA crossovers within site I yield two circular DNA molecules linked together as catenanes. **C.** The (–) supercoil dependence for *in vitro* recombination is shown by the left scale [31] and the *in vivo* supercoil dependence for the *Salmonella* chromosome resolution is shown on the right scale [4]. *In vivo* recombination is aided by nucleoid associated proteins that make the deletion efficiency of a 9 Kb segment occur at 50% when $\sigma_D = -0.030$ (blue arrow) and the deletion efficiency approaches 100% at $\sigma_D = -0.043$ (red arrow).

A.

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B.

DNA Topology of the *Salmonella* *rrnG* Operon

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FIGURE 2.

Supercoil Dynamics in Theory (A) and *in vivo* in the Chromosome of Actively Dividing *Salmonella* Cells (B). **A.** The twin domain model of Liu and Wang [24] predicts that RNA synthesis generates two states of opposite handed supercoiling during steady state transcription. (–) supercoils are predicted behind RNA polymerase whereas (+) supercoils are proposed to exist downstream of the transcription terminator. **B.** Supercoil-sensitive Resolution Efficiency (R.E.) across the *rrnG* operon show very little reaction (1%) unless transcription is diminished by addition of rifampicin or entry into stationary phase [19]. R.E. of 9 Kb regions upstream of the *rrnG* promoter show a higher than average superhelix density while the supercoil level in a downstream 9 Kb regions is depleted in WT cells. The impact of the *gyrA215^{TS}*, *topA715*, and *gyrB1820^{TS}* mutations are shown below the WT.

FIGURE 3.

Resolution Assays Around the *Salmonella* Typhimurium Chromosome. Recombination efficiencies at 8 locations were measured for WT and TS mutants of Topo IV (*parE206*^{TS}) and DNA gyrase (*gyrA209*^{TS} and *gyrB652*^{TS}). Regions corresponding to the *E. coli* macrodomains [32] are color coded: Green, Ori Domain; Black, Right Unstructured Domain; Red, Right Domain; Purple, Ter Domain with red hatches showing *matS* sites; Blue, Left Domain; and Black, Left Unstructured Domain. Arrows outside the circle show the direction of replication fork movement in Replichore 1 (brown) and Replichore 2 (pink).