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Importance of disentanglement and entanglement during DNA replication and segregation:

Comment on: “Disentangling DNA molecules” by Alexander Vologodskii

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In the Vologodskii review [19], the accompanying comments, and many other publications, there has been considerable effort to analyze the actions of type II topoisomerases, especially with regard to “topological simplification”[4]. Whereas these efforts could be characterized as a battle of the models, with each research team arguing for their version of how it might work, each specific kinetic concept adds important considerations to the fundamental question of how these enzymes function. The basic tenet, however, of what is called the “hooked juxtaposition model [1],” is not a modeling aspect, but is simply a geometric mathematical fact.

When strings have physical and geometric qualities (such as thickness, stiffness, or curvature), the geometric information about any given conformation of the strings carries information about their topological state. In particular, we found that the local geometry of physical strings at juxtapositions can determine entanglement (to varying degrees and depending on the nature of the strings). This general mathematical relationship was discovered based on analytical considerations in the original paper that proposed the hooked juxtaposition hypothesis [1], has been validated in computational studies [2,3], and holds true for all sorts of strings: randomly linked paper clips, earphone cords, and DNA, to name a few.

Whether cells make use of the discriminatory information present in the geometry of DNA juxtapositions, and, if so, how the information is read and acted upon by type II topoisomerases, is and ought to be a subject of debate and discussion, including this view by

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Vologodskii. His two-step recognition proposal provides a possible method for reading hooked juxtapositions. Chan and Liu in their comment [this issue], however, argue that computationally there is little difference between a two-step and a one-step method, provided certain kinetic conditions are met. More biochemical analyses are needed to test these conditions.

Although unable to attain the rate of unlinking of the experimental results of Rybenkov [4], kinetic proofreading [22] is another possible way to read juxtaposition geometry. The geometrical constraints of hooked juxtapositions lead to greater persistence under fluctuations than unhooked ones. Thus, inasmuch as kinetic proofreading is successful as a model, it is also consistent with hooked juxtaposition theory.

As we discussed previously (Fig. 1 of [1]), DNA supercoiling tends to localize entanglements into hooked juxtapositions. In general, any sort of packing, which effectively ‘shortens’ the DNA, tends to localize entanglements into hooked juxtapositions. This localization is likely part of what happens in chromosome separation—packing daughter chromosomes makes their entanglements distinct.

Structural studies have shown that the DNA strand is bent when bound to some type II topoisomerases [23]. One could wonder to what extent this bending was there before the enzyme arrived. The bending may be too severe to be independent of type II topoisomerase action, but this notion fails to address whether it would be easier for the enzyme to attach if the DNA were already somewhat bent, as it might be in a hooked juxtaposition. Meanwhile, a hairpin in the string is more likely to catch another string if it is near a hooked juxtaposition because the constraints of a hooked juxtaposition cause the nearby section to be sampled more thoroughly under fluctuations. In this sense, then, hooked juxtaposition theory also explains the discernment Vologodskii found in his original hairpin model.

In spite of a thoughtful review, there are a few inaccuracies we wish to point out. Vologodskii claimed that the hooked juxtaposition model “has an important drawback...” (that) “The experimental data show that the enzymes do not bind two DNA segments simultaneously as the model assumes.” This statement is incorrect for two reasons. First, type I and type II topoisomerases, as measured using a wide variety of experimental techniques, simultaneously bind two DNA helices ([24–27] and references therein). Second, this assumption is not necessary and therefore was not made in the original model [1]. Vologodskii further argues that type II topoisomerases “would wait nearly forever trying to catch a strongly hooked juxtaposition.” He presents no analytical, computational, or experimental evidence to support or explicate this assertion. He thus recognizes that DNA strands juxtapose, and that, depending on the situation (for example supercoiled DNA), numerous such juxtapositions exist.

There is general consensus that type II topoisomerases disentangle daughter chromatids enabling them to be pulled apart during cell division. One of the key observations of the hooked juxtaposition theory is that physical strings under any ‘force,’ whether it be the volume exclusion of knotting or linking or an actual pulling on the strings, all entanglement is in the same geometry: hooked juxtapositions. So whereas one might argue about the

biological significance of topological simplification of relatively small circles, it is hard to argue against the utility of an unlinking enzyme acting at hooked juxtapositions considering the multitude of DNA entanglement problems.

The “remove all DNA links at all costs” *a priori* assumption [4] that underlies much of Vologodskii’s (and others’) discussion does not take into account important facts about chromosomes in living cells. Movement of the DNA replication complex (the replisome) in relation to the antiparallel double helical template is generally modeled in one of two extreme ways. In one model, the replisome is viewed as a moving barrier that drives positive supercoiling ahead of it to levels that would arrest replisome progression without topoisomerases. In the other model, the replisome rotates freely around the helical groove, consequently intertwining replicated DNA duplexes around each other (behind the replisome) at one wrap per 10.5 base pairs [5]. The resulting intertwined replicated DNAs are called “precatenanes” [6,7]. A hybrid of these extreme models is one in which the replisome drives high levels of positive supercoiling tension that then rotates the entire replication fork to intertwine the two replicated duplex DNAs, again resulting in precatenanes [6,7].

Despite the inherent challenges of measuring DNA topology in living cells, there is now strong evidence that precatenanes normally form behind replication forks in cells in all domains of life. First, all cells (or hijack from their hosts, in the case of some viruses) contain dedicated topoisomerases to remove positive supercoils and different dedicated topoisomerases to remove precatenanes. Second, the type II topoisomerases, which are essential for life in all cells and during each cell cycle [8], are tightly associated (physically localized) with the replication fork for the duration of the replication period, and not just at the site of replication fork convergence where maximal topological strain would be thought to occur [9,10]. Third, inhibiting decatenation by type II topoisomerase prevents chromosome segregation but does not prevent replication fork movement. This clear separation of function has been shown in frog oocyte extracts treated with a topoisomerase II inhibitor, the anti-cancer drug etoposide [10], in yeast using a conditional type II topoisomerase under non-permissive conditions [11], and in *E.coli* by the depletion of topoisomerase IV (the *E.coli* decatenating type II enzyme [7]) activity using an antibiotic topoisomerase inhibitor, a fluoroquinolone, or conditional mutants [7,12,13]. Fourth, the observed phenomenon whereby replicated “sister” chromosomes are held together (chromosome cohesion) until cell division is at least partially reliant on precatenanes [14,15]. Once thought to be exclusively caused by a kind of molecular glue (cohesin protein), cohesion persists through much of the cell cycle in eukaryotic cells in the complete absence of cohesin protein [reviewed in [16]]. Cohesion even occurs in *E.coli* cells, which do not contain a known cohesin protein [[12] and references therein].

Precatenation thus appears to have important biological roles involving the timing of DNA segregation and cell division [10], and is not merely a side effect of DNA replication that causes topological problems to the cell. Precatenanes may also prevent closely-spaced recombination events (so-called “crossover interference”), an important process in meiosis [17]. Finally, by converting left-handed positive supercoils ahead of the fork to right-handed precatenanes behind the fork, precatenation might alleviate positive supercoiling, which

would otherwise slow fork progression and spur potentially toxic and mutagenic double-strand breaks at stalled replisomes [18].

Precatenanes are long-lived in *E.coli* cells, often persisting well after the DNA replication period. So how do these links escape too-early decatenation by type II topoisomerase? One possibility is that their right-handed crossings are naturally resistant to type II topoisomerase because left-handed positive supercoils are the preferred substrate of the relevant type II topoisomerases. If so, then what triggers the apparent programmed-like timing for chromosome decatenation just prior to cell division? Precatenane unlinking is strongly tied to condensation of the chromosome, which occurs at late prophase [14,16], a time in the cell cycle when chromosomes morph from “cottony blobs” into highly condensed strings. Such a dramatic change in chromosome structure could create tension, which could result in hooked juxtapositions, a better substrate for the enzyme.

In bacteria, cohesion is strongest (longest lasting) where a protein called SeqA binds [12]. With the type II topoisomerases, SeqA tracks with the replisome in cells. In a test tube, purified SeqA oligomerizes into long DNA-bound filaments that restrain supercoils and modulate topoisomerase IV unlinking [[20] and references therein]. Cells overexpressing the genes encoding topoisomerase IV or in which SeqA is depleted exhibit dramatically slowed chromosome separation (Fig. 1B) and dramatically arrested cell division [12]. These observations indicate that premature unlinking by overproduced type II topoisomerase or the lack of SeqA constraining precatenanes results in premature unlinking of precatenanes and that this early unlinking is detrimental to cells. A model based upon these data proposes that SeqA binding may alter DNA juxtaposition geometry of precatenanes from type II topoisomerase-reactive hooked juxtapositions to unreactive free juxtapositions [1] (Fig. 1A), providing a way for cells to control the switch from linked to unlinked chromosomes triggering segregation.

The surprising experimental results of Rybenkov et al., on their own merit, warrant a thorough understanding [4]. Before these results, many people thought that topoisomerases were generally “blind” to the DNA they were cutting and passing – that their action was as likely to link as to unlink DNA. As noted by Vologodskii, Rybenkov’s substrates were simple, non-topologically closed rings with no supercoiling, no precatenanes, and no SeqA – an idealized form far from their biological context. Nonetheless, that some purified type II topoisomerases in the test tube preferentially unlink these DNA rings more often than they link them gives hints to what these enzymes are capable of doing. And no one has refuted the validity of these experiments; they only challenge (and, in so doing, sharpen) the models that seek to explain the results of these experiments.

In summary, various aspects of type II topoisomerase mechanism (atomic understanding of various enzymes bound to DNA, number of ATPs hydrolyzed per strand passage event, and more) have been fairly well defined. Yet, even after over 40 years, exactly how these enzymes carry out their jobs – in the context of a reconstituted system in the test tube or in living organisms – remains to be elucidated. It is gratifying that twelve years after we first advanced hooked juxtaposition theory it is so resoundingly embraced. One must remember, however, “all models are wrong but some are useful” [28].

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Fig. 1. A possible molecular switch to control chromosome cohesion in *E. coli*

(A) Hooked juxtapositions (HJ) are converted to type II topoisomerase-resistant free juxtapositions (FJ) by binding of SeqA protein, a methylation-dependent DNA binding protein. At the appropriate time, SeqA binding is reversed by DNA methylation, converting free juxtapositions back into hooked juxtapositions, which triggers decatenation by topoisomerase IV. (B) At time “-10 minutes”, *E. coli* cells were “born” (divided off from each other and captured by the “baby machine” method, [21]). Over time, cohesion was monitored for several different locations in the genome. In normal (wild-type (WT)) cells, the replicated regions are held together (red region in the graph) until the point where chromosomes become visibly separated (green region). Ten replication events were quantified; best fit for the average of these data is shown as a dashed line. Premature cohesion release caused by decatenation by overexpression of the genes encoding topoisomerase IV or by the deletion of the *seqA* gene caused a 70% decrease in chromosome segregation. Data are from [12].