PDFR: they found that all of the lLN_vs, one of the LN_ds, seven of the DN1s and one of the DN1 pacemaker neurons co-label with *per*-driven GFP. Anti-PDFR-N thus labels a higher proportion of pacemaker neurons than anti-PDFR-C (Figure 2). While the reasons for these differences are not yet clear, they should be resolved in short order. Further work will focus also on the non-clock PDFR neurons that constitute the pacemaker output pathways.

We can now expect that putative anatomical feedback loops within the pacemaker circuit will be revealed as well as downstream PDF-responsive targets including circuits that mediate locomotor and geotaxis behaviors. Other work provokes questions regarding synchronization of circadian physiological outputs. Genetic manipulation of subsets of clock neurons along with studies in which unusual environmental light-dark conditions that induce complex behavioral rhythms with multiple periods and altered clock cycling in the pacemakers of flies and mammals reveal the importance of synchronizing multiple clocks *in vivo* [6–10,15]. Recent electrophysiological evidence provided by extracellular recordings in the cockroach brain suggests that PDF acts to synchronize assemblies of target neurons [16]. This work has intriguing parallels to electrophysiological studies in mammals [17,18]. Future electrophysiological analysis combined with behavioral genetics will reveal the detailed mechanisms of PDF actions within the pacemaker circuit.

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DNA Sliding Clamps: Just the Right Twist to Load onto DNA

Two recent papers illuminate a key step in DNA sliding clamp loading: one reveals the structure of the PCNA clamp wrapped around DNA — still open from being loaded — while the other finds that the clamp may assist this process by forming a right-handed helix upon opening.

Daniel Barsky[1] and Ceslovas Venclovas[2]

DNA sliding clamps were first characterized as DNA polymerase processivity factors: without their presence, cell division would be inconceivably slow; replication of long stretches of DNA would be hopelessly inefficient because DNA polymerases tend to fall off the DNA after elongating a strand by just a handful of bases. By tethering the polymerase to the DNA, such processivity factors enable the polymerase to add thousands of bases in a few seconds without detaching from the DNA [1,2]. The term ‘sliding clamp’ aptly describes a protein that, while holding on to the polymerase and binding tenaciously to DNA, can still travel vast distances along the DNA. Direct observation of the remarkable ring-shaped structure of the dimeric polymerase III β subunit (or β clamp) [3] provided a beautifully simple mechanism for sliding without falling off: the protein can form a closed ring around DNA and slide along like a washer on a very long screw.

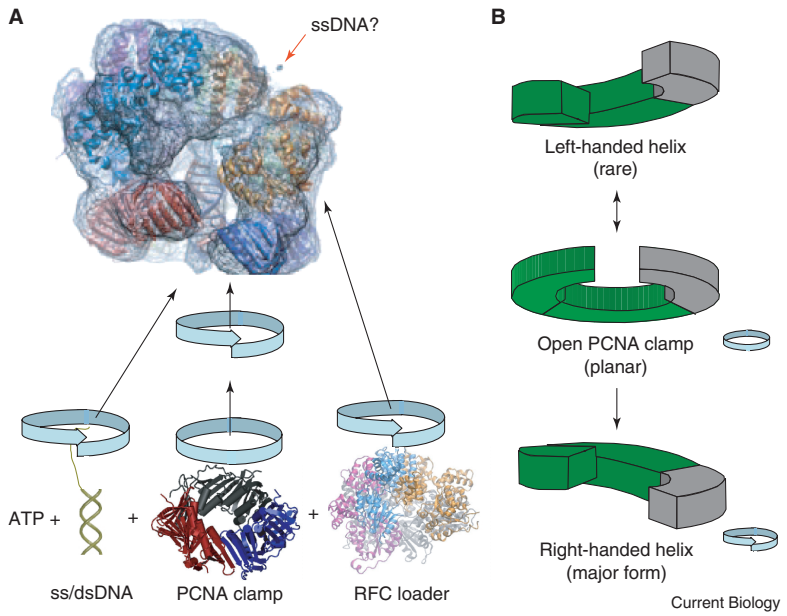

Figure 1. Getting a clamp around DNA.

(A) At the top is a recent EM structure of the open PCNA clamp, RFC clamp loader, and single-stranded (ss)/double-stranded (ds)DNA [13]. Below are the separate components, all of which are right-handed helical, except for the PCNA clamp. Upon opening, however, molecular dynamics simulations suggest that the PCNA clamp also adopts a right-handed helical conformation. (B) Schematic illustration of the dimeric PCNA clamp molecular dynamics simulations with the third 'shadow' subunit shown in grey. Most molecular dynamics simulations displayed a planar to right-handed helix transition [14].

Since their discovery as processivity factors, DNA sliding clamps have been found to be involved in almost every process dealing with DNA metabolism, including replication, modification and repair [4–7]. The major clamps associated with DNA replication are the dimeric β clamp in bacteria and the trimeric proliferating cell nuclear antigen (PCNA) clamp in eukaryotes and archaea. These clamps have been found to interact with a wide variety of proteins and protein complexes, and have been described as molecular tool-belts or moving platforms [8,9]. Yet, many mechanistic details of how these ring-shaped clamps end up wrapped around DNA are still not known. Nature might have invented a clamp that spontaneously opens (like the letter C), binds to DNA and then, stimulated by the presence of DNA, simply closes into a ring around the DNA; but this does not happen.

Instead, all cellular DNA sliding clamps are stable in the closed ring form [3,10,11], and consequently they must be actively loaded onto DNA at double-stranded/single-stranded DNA junctions, placing them at exactly the right place for DNA replication and repair. This process is accomplished by an ATP-fueled clamp-loader protein complex: replication factor C (RFC) in eukaryotes and archaea, and the γ-complex in bacteria. Once on the DNA, the clamp reseals around the DNA and the clamp loader is ejected. Implicit in this description of loading are two postulates: that the clamp actually forms a closed ring around DNA, and that, at some prior moment, the clamp ring must be somehow opened. While there has been ample evidence for this, direct observations of these two points have been lacking. For example, last year a crystal structure was determined of the PCNA clamp bound to the RFC clamp-loader complex in the presence of a non-hydrolysable ATP analogue, but the clamp remained closed [12].

Most recently, however, electron microscopy (EM) has afforded a glimpse into the process of PCNA clamp loading onto DNA [13]. In the reported images, two features stand out: first, the DNA helix was clearly observed running through the middle of the PCNA clamp and into the RFC clamp loader and, secondly, the clamp is open and resembles a right-handed lock- or spring-washer (Figure 1A). Thus, the two postulates above have now been directly confirmed. Because of the limits of EM resolution, no atomic details about molecular interactions within the complex could be observed, but an accompanying modeling and simulation paper [14] provides some important clues as to what it takes for PCNA to adopt such a distorted shape. Molecular dynamics simulations of dimeric PCNA (normally a homo-trimer) imply that, once opened, the clamp tends to move toward the spring-washer conformation similar to that seen by EM (Figure 1B). Analysis of the simulations also suggests that most of the flexibility occurs at the intermolecular interfaces. These two papers [13,14], therefore, are mutually consistent in furthering our knowledge of the mechanics of DNA sliding clamps.

In the first of the new papers, Miyata *et al.* [13] report averaging nearly 20,000 EM images of the archaeal RFC–PCNA–DNA complex to achieve high enough resolution (12 Å) to identify unambiguously the relative positions, orientations, and even the conformational state of the biomolecular complex. Even in the absence of atomic detail, this EM structure is the first view of a double-stranded DNA helix (a 30-mer strand primed by 11 bases) running through the middle of a clamp. Using biotin/streptavidin labeling, the authors were also able to infer an approximate exit path for single-stranded DNA out of the clamp loader. The way out for DNA may lie in the RFC crack lined up, mostly it seems, by individual domains of the large RFC subunit (red arrow in Figure 1A).

Perhaps most interesting is that the PCNA clamp is cracked open by about a quarter the width of DNA (the authors report 5 Å) and appears flush with the right-handed surface formed by nucleotide binding domains of the RFC clamp loader. This is in stark contrast with the features of the homologous RFC–PCNA complex

from yeast [12], where the PCNA clamp remains flat and closed and only partly attached to the RFC clamp loader. In both structures, the clamp loaders are in the ATP-bound state, which is known to result in clamp opening upon engagement [15,16], yet the clamp is open only in the current EM structure. Do these complexes represent different steps in the clamp loading or does the presence of the DNA make a critical difference? Or could the artificiality of either the protein crystallography or of negative-stain EM account for the difference?

There could be a number of explanations, but a simple, hitherto unaddressed issue might explain the incompatibility of the two conformations of the RFC-bound PCNA clamp. Apparently, the crystallized form of PCNA (in the yeast RFC–PCNA complex) has an extra sequence tag of 25 residues at the amino terminus [12]. Although parts of this additional tag are significantly disordered (and therefore unresolved), at least part of it can be seen binding to the neighboring subunit, effectively bridging across one of the interfaces, creating significant hydrophobic contact and likely stabilizing the interface. It has been thought that the mechanism of clamp opening depends on the loader destabilizing one of the subunit interfaces, a notion supported by the crystal structure of a complex of one loader subunit (δ) from *Escherichia coli* with half the β clamp [17]. Although the extra amino-terminal residues may not prevent the clamp from being actively loaded, it is certainly possible that the energy provided by these contacts is sufficient to bias the clamp toward the closed state, preventing it in the all-or-none process of crystal formation from opening fully and engaging the spiral surface of the clamp loader.

The second paper [14] illuminates the question of how the PCNA clamp might get into the observed open conformation. The authors investigated the dynamics of an open clamp by performing a series of molecular dynamics simulations on the PCNA clamps from humans, yeast, and an archaebacterium for a total of nearly 100 nanoseconds of simulation time. The simulations were begun with the removal of one subunit from the closed PCNA homo-trimer, a tactic that might seem quite drastic, except that it avoids having to otherwise modify or distort the clamp. The dynamics of the dimeric form of the various PCNA structures were then analyzed for gap widening in the plane of the clamp (lateral motions) as well as out-of-plane motions. The analysis was related to the full trimeric clamp by the addition of the 'shadow' of the missing subunit, in the place it would be if it moved relative to the second subunit the way that the second subunit moves relative to the first. The analysis revealed that the opened clamp opened further, analogous to previous molecular dynamics simulations on a monomer of the β clamp [17].

Unlike the β clamp monomer simulations, these dimeric PCNA simulations allow an analysis of the motions between subunits. In the simulations, the flexibility of PCNA appeared mainly in the subunit interfaces rather than in the domain interfaces. Perhaps more surprising was that in seven out of nine simulations, out-of-plane motions tended toward a right-handed helix. This is a remarkable result, suggesting that the clamp has evolved to cooperate in the loading process. The emerging picture is that the clamp loader in the presence of ATP binds to the clamp and interrupts one of the interfaces between the clamp subunits, causing the clamp to 'spring open' into a right-handed helix, which is then stabilized further by binding to the clamp loader. At that point, the open clamp can be loaded onto DNA, after further widening (at least beyond the 5 Å observed in the EM structure) to allow double-stranded DNA to enter, or possibly letting only the single-stranded portion of DNA to squeeze in. Exactly how the negatively charged DNA enters the open clamp may also depend strongly on the details of the charge distribution of the clamp and clamp loader complexes.

It may be useful to clarify what is meant by the clamp 'springing open' or being 'spring-loaded' — terms first introduced to rationalize the reduced curvature of a single subunit of the β clamp, relative to the full dimeric β clamp, consistent with molecular dynamics simulations of the β monomer [17], and also used to refer to the gap widening of the PCNA dimer observed in the latest molecular dynamics simulations [14]. What is not meant is that the closed clamp is like a compressed spring, higher in energy than an open clamp. In fact, as both PCNA and β remain closed in solution (as well as in the crystals so far resolved) [3,10,11], the closed ring must have the minimum energy conformation. This minimum, however, may be divided into two parts: one part due to the favorable contacts at the interfaces between subunits, and the second due to the rest of the latent energy of the conformation (Figure 2). What has

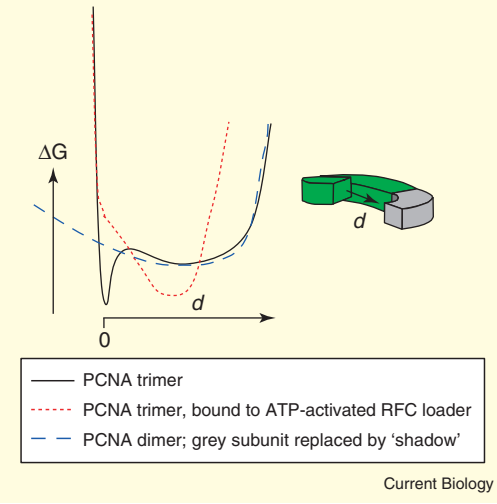

Figure 2. Sketch of the free energy landscape (ΔG) versus gap opening distance *d* for the PCNA clamp.

When *d* = 0, the interface stabilizes the closed trimeric PCNA ring (black, solid), while the RFC loader stabilizes the open ring (red, dotted). With no third interface, molecular dynamics simulations of the PCNA dimer display a tendency toward the open ring (blue, dashed). This graph is meant to be illustrative; the exact shapes, relative heights, and minima of the curves are not known.

been observed by molecular dynamics simulations is that, once the interface is removed (by removing one of the subunits), the structure relaxes and adopts a more open, less-curved, tending-towards-right-helical shape. The implication, of course, is that if the loader is able to abolish one of the interfaces between clamp subunits, the clamp will then open further without requiring an *additional* energetic contribution from the loader.

This, however, raises the question that if the loader can, in the presence of ATP, bind to the clamp strongly enough to outcompete the favorable contacts between clamp subunits, how is it able to release the clamp? Although the details are not yet clear, this must be the role of ATP hydrolysis. It is likely that, just as the ATP binding to the RFC clamp loader is a coordinated process [16,18], ATP hydrolysis is also a coordinated process, as was shown for the bacterial γ-complex [19]. In any case, hydrolysis of ATP and release of ADP should restore the initial orientation of amino-terminal domains in the clamp loader [20], making this orientation incompatible with the high affinity for the clamp and thus allowing the loaded clamp to disengage.

As discussed above, new data from electron microscopy and insights from molecular dynamics simulations are rapidly completing the picture of how clamp loading occurs. When it was first realized that sliding clamp loaders involve a complex of five proteins that must open the clamp, load it onto DNA and release itself in an ATP-driven process, the mechanistic details were expected to be quite complicated, and they have lived up to those expectations. Somewhat surprising, however, has been that the seemingly passive clamp would also hold such fascinating features as the conformational agility observed by EM and by molecular dynamics simulations. As the role of DNA sliding clamps continues to expand from processivity factor to central player in DNA metabolism, it is also being realized how finely tuned these proteins are for binding other proteins, for tenaciously grabbing and sliding on DNA, and now it seems for assisting in the loading process. It might well be that sliding clamps still have surprises left to be discovered.

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Coevolution: The Geographic Mosaic of Coevolutionary Arms Races

Coevolutionary arms races between species can favor exaggeration of traits for attack and defense, but relentless escalation of these arms races does not necessarily occur in all populations.

John N. Thompson

Coevolution between species sometimes results in astonishingly exaggerated traits. Some newt species harbor a very high level of tetrodotoxin that protects them from most predators [1], and some plant species have complex suites of chemical and anatomical defenses that protect them from a diverse array of herbivores and