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Supercoil-driven DNA structures regulate genetic transactions

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1. ABSTRACT

DNA is a living molecule, writhing, twisting and bending in response to the physical forces applied to it by genetic processes. Twisting and untwisting of the double helix by powerful molecular motors generates, at least transiently, high levels of torques. Although under relaxed conditions the double helical B-form is the predominant conformation of DNA, in response to physical stress, B-DNA strains inhomogeneously, adopting a variety of alternative structures. These structures are the sites of genetic damage that increase the fragility of the genome, but they may also participate in physiological processes performing functions not achievable using conventional duplex. The dynamic response of DNA to supercoiling forces contributes to control of genes such as *c-myc* whose physiological levels must be precisely regulated.

2. THE STRUCTURAL DIVERSITY of DNA

In the era of systems biology, the informational role of the genome in the pathogenesis of disease has been preeminent. From this perspective, events that alter nucleic primary structure--substitutions, insertions, deletions, rearrangements, translocations, etc.-- provide the pathogenetic basis of inherited diseases through the direct miscoding of polypeptides or through the mutation of cis-elements and consequent misspecification of regulatory information. Covalent modifications of DNA have long been known to provide an additional epigenetic layer of gene control (1), and disturbances in the placement and maintenance of these modifications are also associated with pathology. More recently the arrangement of nucleosomes and the covalent modification (or de-modification) of their constituent histones have been shown to play key roles in

determining gene activity and to contribute to the transmission of epigenetic states. Alternative DNA conformations provide another source of structural diversity. Non-B DNA structures include single-stranded DNA (2), H-DNA (3), Z-DNA (left handed double helix) (4), G-quadruplex (5, 6), triplex DNA (7), slipped-strand DNA (8), sticky DNA (9), S(stretched)-DNA (10) and cruciforms (11). Although the properties and characteristics of these structures *in vitro* have been well described, their relevance to genetic processes, and even existence, *in vivo*, have often been questioned. Because these structures are relevant to pathology, then they must form at least occasionally *in vivo* and disturb genetic processes by interference with replication, transcription, DNA-damage repair, recombination or by promoting genetic damage, translocations, DNA breaks, mutations (9, 12-19). If important for normal physiology, then these alternative structures must form frequently in association with the proper execution of regulatory and biosynthetic programs. Each alternative structure must compete for its immediate and evolutionary existence against B-DNA. The global thermodynamic stability of the double helix coupled with its large coding capacity, and evolvability provide resistance to the formation of these structures at the level of the single cell, and provide backward pressure against the global expansion and fixation of these alternative conformations on an evolutionary scale. Each of these conformations has specific sequence and chemical (ionic strength, pH, metal, etc.) requirements to support prolonged stability. So the existence, persistence, and in some cases the expansion of these structures throughout the metazoan genome indicates either that they have evolved to fulfill physiological niches where B-DNA will not easily substitute, or that they are not sufficiently deleterious to be scrubbed from the genome by natural selection.

How do alternative structures form *in vivo*? What are the intracellular parameters that favor alternative DNA conformations? Thermodynamic and kinetic barriers prevent large segments of the genome from adopting non-B conformations (20). The first pre-requisite step for the formation of these structures is destabilization of the native duplex. The incremental or bulk breaking of base pairs, followed by some measure of strand separation must precede the reorganization and reorientation of both the phosphodiester backbones and of the bases to the configurations that are characteristic of each structure. Destabilization of B-DNA *in vitro* is accomplished thermally, chemically using denaturants, and electrostatically by removing the counter-ions that otherwise would shield the phosphates on the complementary strands from repelling each other (21, 22). These three means of helix destabilization are largely unavailable in most organisms, though extremophiles have evolved special adaptations both to stabilize and destabilize B-DNA. Though the double helix is dynamic and breathes at ambient temperatures of most poikilotherms and at the physiological temperatures of homoiotherms, this breathing is short lived and the flickering helical instability under native conditions, generally does not favor the formation of alternative structures. The same chemical conditions that denature DNA also denature proteins and so would not be available *in vivo*. Similarly, physiological ionic conditions promote B-DNA stability. Thought is difficult to envision mechanisms how thermal, chemical or ionic insults on DNA structure could be focused just to particular regions of the genome, the response to these insults could be controlled by variations in sequence and base composition would compel an inhomogeneous response to these stresses. For example, the low melting temperature of AT-rich stretches of DNA might have the capacity under some circumstances to undergo a localized conformational change in response to thermal stress. Though AT-rich segments functioning as thermosensors have not been reported, intriguingly an RNA molecule HSR1 has been identified as the thermosensor of the heat shock response. Whether HSR1 undergoes a conformational change in response to thermal stress has not been reported (23).)

3. WHAT FORCES PROMOTE STRUCTURAL ISOMERIZATIONS?

Transcription and replication employ powerful motors to move relative to chromatin; they develop forces even larger than myosin (though over a smaller step-size) (24). If transcription occurs at fixed RNA Polymerase sites, then this process has enough power to push and pull chromatin through nuclear medium. In addition the existence of nuclear actins and myosin indicates the possibility of tugging directly on chromatin fibers (25-29). Anchorage of chromatin to nuclear structures, to actin and myosin and directly or indirectly to cytoskeletal elements provide means to direct mechanical forces to DNA; sequence-dependence, inhomogeneity and anisotropy in the elastic properties of chromatin in principle might all modify DNA structure in response to stress, though these possibilities remain largely unexplored (24, 28, 30-33). The destabilization of B-DNA *in vivo* by molecular motors and enzymes requires energy. Almost all genetic transactions use energy from nucleotide triphosphate hydrolysis to change DNA structure, at least transiently. Helicases and polymerases (RNA and DNA) create unpaired DNA loops and translocate this melted bubble along the helix. In the case of DNA polymerases, alternative DNA structures are possible in the vicinity of the replication fork, where lagging strand synthesis obligatorily creates expanded single-stranded zone (34). In the case of RNA polymerase the RNA-DNA heteroduplex within the active site is 8-9 bp long; thereafter the nascent RNA is guided through the exit channel away from the enzyme, allowing reannealing of the non-template and template strands (35). In this situation, it would appear that the unpaired loop is confined within the enzyme and so there is little exposed unpaired DNA to nucleate the formation, and to expand and become an extended stretch of non-B DNA. However transcription, replication and other genetic processes transiently generate high levels of supercoiling forces that can be transmitted to remote

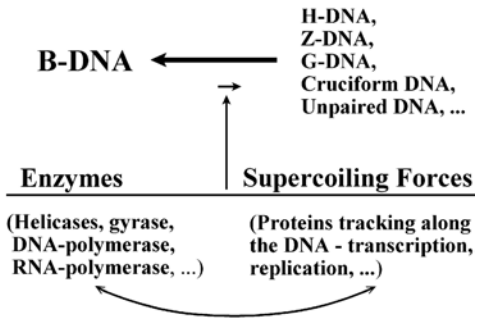

Figure 1. A scheme showing factors involved in the stabilization of non-B-DNA *in vivo*. B-DNA is the canonical right-handed DNA. For non-B-DNA formation special sequence characteristics are usually necessary. Z-DNA is left-handed helix found mostly in alternating purine-pyrimidine sequences. H-DNA is a triple-stranded structure found at purine-pyrimidine sequences of mirror repeat symmetry. It consists of the B-form helix with third strand bound in the major groove and a fourth unpaired strand. Cruciform DNA formed at palindrome sequences consists of two B-DNA arms and two hairpin arms which form a four-way junction. Unpaired DNA formed at sequences which have little similarity except for being AT-rich. G-DNA has been shown to form from tandem repeats of G-rich sequences. It is four-stranded helical structure stabilized by stacking interactions. Note that enzyme-action and supercoiling force are interconnected as they are both involved in DNA-transactions; in the absence of a eukaryotic DNA gyrase, threading of DNA through the active site of a tracking enzyme is the most common mechanism for generating torsional stress.

sites where they may promote structural transitions (Figure 1) (36, 37).

Fixing the ends of a segment of DNA creates a topological domain containing a fixed number of helical turns. Within this domain $L_k = T_w - W_r$, here L_k, the linking number, is an integer reporting the absolute number of times one strand is interlinked with its partner (20); T_w is the number of helical turns appropriate if the segment between the two sites of fixation were mechanically unstressed B-DNA; W_r is writhe (supercoils), a descriptor of the three dimensional trajectory of a vector parallel to and traveling along the central axis of the double helix within the boundaries of the fixed DNA segment (38). The ends of a DNA segment may be fixed by many devices *in vivo* to create a topological domain (39-43). Interactions between DNA-bound proteins define topological domains. DNA-looping, whether short range or long-range juxtaposes non-adjacent segments of DNA. The protein-protein interactions closing these loops provide a barrier to prevent the spinning of the bounded segment around its main helical axis. Attempts to perform such rotations wrap one region of the double helix around another resulting in braids--plectonemic supercoils (44). Supercoils generated within such a domain have several fates. (a) They accumulate until the torsional stress is strong enough to sever the protein-protein bridge thus releasing the supercoils into a larger embracing domain, (b) they accrue until the accumulated torsional stress matches the torque applied by whichever enzymatic machinery (for example polymerases or helicases), causing the latter to stall. (c) A toposiomerase (usually a topoisomerase I or II) removes the supercoils. It is important to note that a topoisomerase must be physically located within the same topological domain as the torsional stress it is targeting. Internal protein-protein loops within a topological domain could create subdomains insulated from the action of a topoisomerase in the embracing domain. (d) DNA damage creates a free end leading to the release of the torsional stress. 2) Fixation of DNA to an immobile cellular structure sets a topological boundary. Anchoring DNA or chromatin to nuclear pores or nuclear matrix at two sites defines a topological domain (45). Torsional stress in these domains would be handled in the same manner as in domains define by a protein-protein bridge. RNA has been implicated as a component of chromatin and whether held in place by protein interactions, or direct RNA-DNA interactions, may also provide the link to close a topological domain. 3) Of course covalent closure of a segment of DNA with itself defines a topological domain, i.e. a DNA ring such as a plasmid or episome. Another example of self-made domain organization of DNA is dynamic association of two long GAA•TTC repeat sequences in one DNA molecule (9)

4. RESTRAINED VS. UNRESTRAINED SUPERCOILS

Two sorts of supercoils might cohabit a topological domain, restrained and unrestrained (38). Each nucleosome is wrapped by 147 bp of DNA 1.7 times. Although this wrapping would be expected to yield 1.7 negative supercoils, simultaneously over-twisting of the DNA spooled upon the nucleosome and/or accommodation by directionality of the crossing of the DNA arms entering and exiting the nucleosome, slightly increases the total number of helical turns within the 147 bases to offset slightly the -1.7 supercoils for a net of -1.05 supercoils per nucleosome (46-48). These supercoils are restrained (fixed) upon the nucleosome and are the consequence of compacting DNA into chromatin. The energy stored in these coils is unavailable to do work unless this DNA is released from the nucleosome. So a linear unstressed segment of chromatin includes -1.05 supercoils per nucleosome. The stress from over- or under-twisting the double helix away from this neutral position would be expected to be accommodated principally within the linker regions separating the nucleosomes (the alternative would require perturbation of the stereochemistry of the DNA-protein interactions of the nucleosome). The torque that results from twisting chromatin away from the neutral position represents potential energy recoverable for work. Within a topological domain, only the unrestrained supercoils are susceptible to rapid removal by topoisomerases (49).

5. SUPERCOILING FORCES REGULATE PROKARYOTIC GENES

Many investigators have assumed that the abundance, distribution and activity of topoisomerases is sufficient to drain all unrestrained supercoils from the

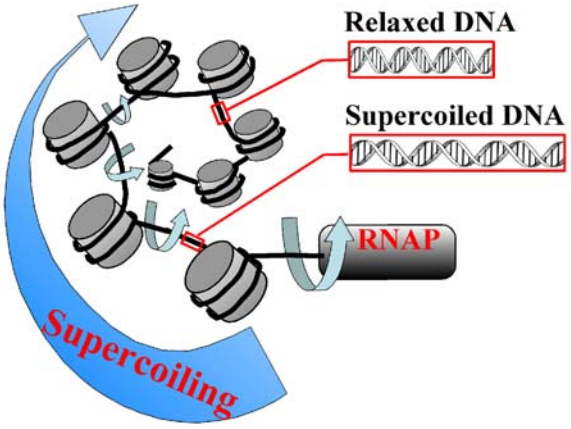

Figure 2. A graphical illustration showing the mechanics of the transmission of the torsional stress through the chromatin fiber. If RNA polymerase (RNAP) is moving from left to right without rotating in 3-D, then due to its helical structure, the DNA must be screwed through the enzyme. The DNA behind the transcriptional machinery becomes undertwisted (negatively supercoiled). This supercoiling might diffuse into upstream region due to highly dynamic nature of DNA-nucleosome core interaction and structural plasticity of chromatin fibers, and must be accommodate by supercoiling in the linker region, rotation of nucleosomes en bloc or by altering the stereochemistry of the histone octamer-DNA complex.

metazoan genome; if so this would be in sharp contrast to the situation in prokaryotes where ongoing genetic activity sustains global torsional stress according to the metabolic state of the cell (42, 50-53). Bacterial DNA is highly supercoiled during the mid-log phase of growth and lessens as the cells approach stationary phase (50, 54-56). This change in supercoiling has important regulatory consequences. rpoS is the sigma factor that replaces sigma 70 as cells proceed to stationary phase; rpoS functions at lower superhelical density than the sigma70 so target expression is tuned to the level of unrestrained torsional stress in the genome (54, 56, 57). Torsional stress in E. coli has also been proven to directly regulate IHF-responsive promoters; competition for melting to relieve torsional stress between IHF-binding sites and promoters determines the level of gene activity (57). In E. coli, negative supercoils propagating behind an elongating RNA polymerase have been shown to drive cruciform extrusion *in vivo* (58-60). The activity of bacteriophage N4 virion RNA polymerase is intimately coupled with superhelical stress to generate the cruciform structures required for promoter recognition and transcription in duplex DNA (61-66). So dynamic supercoils help to maintain proper expression of prokaryotic genes.

6. IS THE MAMMALIAN GENOME FUNCTIONALLY SUPERCOILED?

In mammalian cells the situation is more complicated. Psoralen cross-linking studies (based on the fact that photobinding of psoralen to DNA is proportional to supercoiling density) have revealed that the bulk of genomic DNA is torsionally relaxed and contained only constrained supercoiling (67). However, unconstrained supercoiling (torsionally stressed DNA) was detected in few loci (41, 68-71). The accumulation or dissipation of the torsional stress might be important for genome functioning. Although intuitively, these stresses might be expected to be short-lived, relaxation or removal of torsional stress is opposed by the architecture and arrangement of chromatin and the nucleus. If RNA polymerases are localized to transcription factories and so prevented from rotating around the template, then the double helix would obligatorily be required to be threaded through the active site of an elongating transcription complex (72-74) (Figure 2). This would necessitate the rotation of chromatin segments festooned with sorts of protein-DNA complex. The frictional forces opposing the screwing of chromatin though the active site of an immobilized complex might be considerable. RNA polymerases are among the most powerful motors in the cell generating very high levels of force, so the high levels of torque are achievable by this enzyme would overcome most of the opposing forces (24, 75, 76). Even if the transcription machinery is untethered to the nuclear matrix, its large mass (many dozens of polypeptides in multiple multi-subunit complexes) combined with the frictional drag of primary transcripts encumbered with the splicing machinery, would resist the counterotation about the chromatin filaments that would be required to dissipate the torsional stess. In this situation with high friction, even in a topologically open domain, the continuous or pulsatile generation of torsional stress is offset by slow relaxation to the unstressed state leading to appreciable steady state or pulsatile levels of unrestrained supercoils (77). Local architectural features would modify the degree of dynamic supercoiling. Interactions between DNA bound proteins create topological domains, ranging from mini domains in the case of closely situated factors to mega-domains defined by interactions between factors bound at vast distances such as locus control regions interacting with promoters (78-81). Meanwhile, transcription in eukaryotic cells is often set off by looping between a distantly bound transcriptional factor and a complex of transcription factors at the promoter (82, 83). Formation of an internal domain would augment the effective level of supercoiling in the embracing domain in three ways: First it concentrates any newly generated stress into a smaller segment raising the effective superhelical density. Second, the formation of an internal loop decreases the target size for the removal of torsional stress by topoisomerases. Third, to transmit torsional stress beyond an internal loop, the latter must be rotated en bloc. The friction resisting this rotation dramatically increases with radius of rotation (84). So the mass and architecture of the internal loop could transiently delay or prevent relaxation of supercoiling forces. The dynamics of supercoil propagation would also be sensitive to global parameters such as intranuclear viscosity and conditions that influence the stiffness of DNA such as ionic strength and divalent concentration.

The arrangement of promoters would heavily influence the magnitude of dynamic supercoils. Closely

situated divergent promoters that support bi-directional transcription are a bright feature of the mammalian genome (85, 86). While such an arrangement might provide for co-regulation, the rules determining how transcription factors divide their services between the two promoters have not been elucidated or enumerated. Even if there were no sharing of transcription factors, the activities of divergent promoters might be coupled by dynamically transmitted torsional stress (87, 88). For example, the dynamic supercoils emerging behind two closely situated diverging polymerases would reinforce each other (59). On some negatively supercoiled templates *in vitro*, TFIIH is dispensible *in vitro* (89-91). The energy recoverable from stable or dynamic supercoils would assist strand separation a promoters, essential for pre-initiation complex formation and transcription initiation, bypassing TFIIH helicase mediated melting. The existence of this mechanism is supported by the frequent co-expression of the genes in divergent pairs (85). So transcription from one of a pair of closely situated divergent promoters would generate negative supercoils that would facilitate melting at the second start site, and might be expected to modify or obviate the requirement for TFIIH.

In vitro and *in vivo*, the superhelical density of a DNA segment situated between two active promoters can rise to levels high enough to drive conformational changes at susceptible sites in the DNA (59, 77) and (Kouzine and Levens, unpublished observations). Pre-existing supercoils would also influence the stability and activity of the local chromatin. Pre-supercoiling pays much of the energy expense required to spool DNA on nucleosomes, and lessens the work that needs to be done on the system (largely through electrostatic interactions between histones and DNA) to bend and twist DNA into place. Pre-existing supercoils (whether stable or dynamic) would supplement the supercoiling forces generated by chromatin remodeling machines (92-95).

7. DYNAMIC SUPERCOILS MAY CHANGE THE STRUCTURE OF CIS-ELEMENTS

Dynamic torsional stress might induce DNA-strand separation and form non-B-DNA structures. This fact was used to detect indirectly dynamic supercoiling *in vivo*, using prokaryotic models (58, 59, 96). The direct transmission of twist deformation (torque) over short to mid-range distances has been demonstrated *in vitro* using T3 and T7 polymerases and linear template DNA, a worst case scenario for the transient capture of dynamic torsional stress (77). One kilobase of irrelevant DNA (a segment of the *B. subtilis* sucrase gene) was placed as a strain-sampling sequence between two loxP sites sandwiched by divergent T3 and T7 promoters. In the absence of transcription, the circles excised by Cre-recombinase are relaxed, or more precisely, the distribution of topoisomers is explained by the Boltzman distribution. As the rate of transcription is intensified, the circles excised from active templates capture more and more negative supercoils. The torsional stress in many of the templates crossed the threshold required to drive a conformational change in a supercoil sensitive sequence, the Far UpStream Element, derived from the human *c-myc* promoter. This element is becomes single stranded *in vivo* when the *c-myc* gene is active (2, 97-99), and in fact, FUSE was predicted to be a Stress Induced Duplex Destabilization (SIDD) site based on an algorithm that calculate the thermodynamic stability and statistical probability of melting base-pair by base-pair. SIDD sites melt in response to applied torsional stress depending on particular sequences in which they are embedded.

In its natural setting FUSE is predicted to melt biphasically as the superhelical density advances between -0.045 and -0.065. Melting by torsional stress is more difficult to calculate and it generates a different result than thermal melting for the following reason: Within a topological domain, the melting of any base pair on the central helical axis relieves an increment of torsional stress. When enough base-pairs are melted, the torsional stress is depleted and no further melting is thermodynamically favored. So each base pair is competing for melting with every other base pair within a domain; this fact enables remote, but direct coupling between alternative DNA conformations at widely separated DNA sequences. Because of competition between base pairs for melting, strand separation at SIDD sites is context dependent. The same element that melts easily in one setting, may be rock stable in another where a more easily destabilized competing element absorbs all the resident torsional stress (100-102). (In contrast, thermal denaturation proceeding against a reservoir of heat, allows each base pair to melt according to local features.) Stabilization of duplex DNA at one weak spot, for example by DNA-binding proteins, would transfer stress-induced destabilization to a remote site. In the case of dynamic supercoils the torsional stresses that are propagated vectorially away from a transcribing RNA polymerase, never reach equilibrium within the upstream (or downstream) DNA sequences. The nature of the competition between SIDD sites in a setting with vectorially propagated torsional stress has not been described theoretically or experimentally.

A variety of alternative or competitive conformations are sponsored by high levels of supercoiling (103). Although most of these require at least a nidus of unpairing in order to initiate the conformational change, this melting does not guarantee a structural transition. For example, though the transcription driven melting of FUSE by dynamically generated torsional stress is sufficiently long-lived to bind FUSE-binding protein (FBP) and FBP Interacting Repressor (FIR) via sequence specific recognition, the transcriptionally generated stress is not long-lived enough *in vitro* to promote higher order structural transitions (83). In stably supercoiled DNA in the presence of magnesium, single-stranded FUSE slowly isomerizes into H-DNA; though transcription reactions obligatorily include Mg^{2+}, dynamic supercoils do not drive this isomerization *in vitro* (77). Slow kinetics demand sustained supercoiling rather than pulses of torsional stress to form H-DNA. Between pulses of transcription, it is likely that the strained templates can relax at least in part. To complete the slow isomerization to H-DNA, either the torsional stress must be sustained or alternatively, an unstable intermediate could be kinetically trapped (104).

One particularly plastic element in the *c-myc* promoter is the CT-element, found 250-300 bp upstream of the major P2 promoter and 100-150 bp upstream of the minor promoter P1. Comprised of five repeats of the sequence (C/T) C (C/T) TCCCCA, this element has been shown to adopt a variety of alternative structures *in vitro* including single-strands, triplex, G-quadruplex, and I-DNA (2, 5, 6, 105-108). The formation of any of these structures is predicated upon disruption of normal B-DNA. Though the CT-element is not predicted to be a SIDD site, it has nevertheless been observed to adopt alternative structures in supercoiled DNA *in vitro* and *in vivo* (2, 109). Based on its GC content alone, this region would be expected to conform to stable B-DNA, however, the reiterated sequence and the strong purine-pyrmidine strand bias, allow slipped mismatched structures (20). In these structures, although one strand has shifted relative to the other unpairing a single repeat, the nature of the sequences allows for considerable residual base pairing. The metastability of the slipped structure is conferred by the same GC-richness found in the properly aligned double helix. In order to revert to normal B-DNA, the slipped structure must be broken down. Structural transitions in the CT-element driven by dynamic supercoils have been detected *in vitro* using the unpaired-T reactive agent potassium permanganate; the observed pattern of reactivity seemed to indicate single-stranded character although contributions from other non-B DNA structures could not be excluded (2). Flipping of CT-element between alternative structures may have important physiological consequences because a different set of specific binding proteins is associated with the different conformers. For example, while SP1 binds with duplex CT-element, hnRNP K (a KH-domain protein) and CNBP (a zinc finger protein) bind with the separated purine-rich and pyrimidine-rich strands; so a gene specific response to a ubiquitous transcription factor maybe conferred by regulating local DNA structure (110, 111).

Another segment of the *c-myc* promoter a bit further upstream than FUSE has been predicted to assume Z-DNA (112). This region has been shown to adopt Z-conformation *in vitro* and to cross-link with anti-Z-DNA antibodies within the nuclei of permeabilized cells. If and which proteins bind to this region, under what conditions *in vivo* is not known.

8. THE ELASTIC PROPERTIES of ALTERNATIVE DNA STRUCTURES ARE DIFFERENT THAN B-DNA

Studies of alternative DNA conformations most often study structural transitions in naked DNA. Are these alternative conformations biologically meaningful? Much evidence suggests that these structures are more genetically unstable than B-DNA and so are associated with genetic instability (translocations, insertions, triplet repeat expansion, deletions, etc. (16, 17). Although the genetic damage arising secondary to this instability is highly relevant to disease, the extent to which non-B-DNA participates in normal cellular processes is not known. Whether they exist *in vivo* only rarely and transiently, or endure for long periods is not generally know. The formation of these structures might simply be a by-product of ongoing physiological operations, but these structures might also be functional. How might non-B-DNA contribute to physiological processes? Non-B-DNA might alter the elastic properties of DNA and chromatin. Naked DNA is an intrinsically stiff polymer (113, 114); it resists bending and is even more resistant to twist. Yet *in vivo* there is little evidence that the stiffness of DNA poses a significant impediment to most genetic transactions (115-117). So either the intrinsic physical properties of DNA are different *in vivo* than *in vitro*, or the cell has evolved mechanisms to overcome or bypass the intrinsic rigidity of DNA. Though single stranded loops as small as three base pairs create a hinge greatly facilitating both DNA bending and twisting, such small loops would be expected to revert rapidly to proper B-DNA (113). However stabilized by single-strand DNA binding proteins, melted loops serve as a DNA-protein hinge facilitating all sorts of DNA transactions between flanking sequences (118). Defying the intuitive expectation that single-stranded loops would obligatorily form and be longed-lived at the junctions between B-DNA and alternative structures, the structure at junction of a right handed B-DNA helix with left handed Z-DNA reveals that such junctions can form with an economy of helical disruption. The transition from B to Z is accommodated by flipping out of a single base pair with no other single stranded or disordered regions (119). H-DNA and other triple-stranded and quadruplex structures expose single-stranded segments (these segments themselves might adopt alternative secondary structures) that would be anticipated to be quite flexible, though the rigidity at B-DNA-triplex or B-DNA-quadruplex junctions have not been directly measured. In regions of high superhelical stress where alternative structures form or if these structures are stabilized by other means (such as binding proteins), these junctions might provide the flexibility needed to juxtapose distant elements or to fold chromatin into special higher-order chromatin conformations. All of these structures, including single strands are unfriendly to nucleosomes and so their formation may interrupt or re-position chromatin arrays (83, 120-123). The exclusion of nucleosomes may have implications for a variety of genetic events. Z-DNA is also unfriendly to nucleosomes and Z-DNA formation upstream of the CSF1 promoter helps to keep this region nucleosome depleted during gene activity (121, 122). A role for Z-DNA in regulating gene activity is also suggested by the association of Z-DNA tracts of different lengths near the nucleolin gene with different levels of expression (124).

9. NON-B DNA BINDS PROTEINS

The formation of single stranded segments and other alternative DNA conformations might also function by binding DNA-conformation sensitive proteins. These protein in principle might recognize generic structure, or structure and sequence. Some structure specific proteins are also inherently sequence restricted because other than single-stranded DNA, all of the alternative DNA structures discussed above inhabit a limited sector of sequence space.

For example, Z-DNA requires strictly alternating purines and pyrimidines thus automatically limiting the area of sequences space available for recognition by Z-specific proteins (125, 126).

The stability of B-DNA dictates that unless subjected to physical forces such as torsional stress or acted upon by nucleic acid metabolizing enzymes, or helicases, spontaneously forming regions of non-B DNA are likely to occur rarely and to be short-lived, therefore the stability, kinetics and dynamics of conformational isomerization are likely to be dramatically influenced by DNA-binding proteins. On the one hand proteins that stabilize B-DNA will oppose isomerization to unusual structures, while proteins binding to these structures would favor these transitions. Many proteins have been identified that bind with single-stranded DNA. Although some of these proteins are not sequence dependent and participate in global processes such as replication, others bind sequence specifically. For example, extended regions of single-stranded DNA are found at telomeres and proteins have been identified that recognize these regions (127-129). Opening of DNA due to the translocation of transcription or replication bubbles by RNA or DNA polymerases or due to the propagation and accumulation of torsional stress, transiently exposes short segments of single strands for recognition by sequence specific, single strand DNA-binding proteins. In contrast to binding sites in B-DNA that are available for binding unless masked, the target sequences bound by non-B binding factors are constitutively masked. The ability of proteins to engage alternative structures will depend on the lifespan of these structures, as well as on the concentrations of binding factors. An appropriate binding factor must be in the immediate vicinity to trap evanescent non-B structures. In accord with this notion, factors such as FBP and hnRNP K are abundant proteins (2, 130, 131). The large excess of these proteins may help to insure that they are able to grab onto even a fleetingly open cis-element. In general, though sensitive genetic assays and screens have documented the formation of alternative DNA structures *in vivo*, this same sensitivity may obscure estimation of their frequency of formation or duration of *in vivo*.

One important use of single stranded DNA is to serve as the substrate for activation induced cytidine deaminase (AID), a single strand binding enzyme responsible for immunoglobulin mutagenesis during class switching. The evidence would indicate that AID activity is focused on R-loops between immunoglobulin transcripts and DNA, although high levels of supercoiling may also expose single stranded regions to AID action. Because R-loops are stabilized on supercoiled DNA, in either case stable or dynamic supercoils are likely to contribute to a proper immune response (132-134).

Proteins that bind to single stranded DNA often employ motifs associated with RNA binding; this is not surprising. The definitive chemical distinctions between single stranded DNA and single stranded RNA are limited to the 2'-position of the sugar (deoxy- versus –OH) and the addition of the methyl group at position 5 of uridine's pyrimidine ring, converting it to thymidine. The pucker of the pentose ring is also often different. Other than the thymine-methyl, all of the functional groups available for sequence specific recognition are shared between DNA and RNA. The structural flexibility of single-stranded nucleic acids renders secondary structure distinctions (such as A-form versus B-form with duplex) less reliable discriminators between DNA and RNA. Common protein motifs that bind with DNA or RNA are zinc fingers, RNA Recognition Motifs (RRMs) and hnRNP K homology motifs (KH-motif) (135-140). For each of these families there are individual proteins that may be evolutionarily tuned to bind RNA, DNA or both. The range of proteins and the motifs specifying interactions with Z-DNA, quadruplex or H-DNA is less well enumerated. A variant of the Helix-Turn-Helix (HTH) motif has become specialized for binding with Z-DNA. The details of this interaction were first described for ADAR1, an RNA editing enzyme (de-amination of adenosine to insoine) (141-146). RNA binding occurs through a separate region of the protein. Sequence and structural homology have allowed the identification of structurally related Z-DNA binding proteins. The structural divergence of Z and B-DNA apparently precludes common recognition by a single HTH motif. Although there are multiple proteins that bind sequences capable of forming G-quadruplex in while in duplex or single-stranded, there is a paucity of proteins that actually interact with these sequences while quadruplex. Perhaps the best example is the TIE-alpa/beta pair; TIE-alpha binds the loop at the ends of the quadruplex and helps to recruit TIE-beta to the structure (147).

10. *C-MYC* EXPRESSION IS REGULATED BY STRUCTURAL TRANSITIONS IN PROMOTER DNA.

So supercoiling forces and nucleic acid metabolism at least transiently develop foci of non-B DNA, and there are DNA binding proteins that interact with these non-cannonical structures. But what do these proteins do? The most comprehensive view of the role of non-B-DNA and associated factors for gene regulation in mammalian cells has been developed on the human *c-myc* promoter. *c-myc* is a helix-loop-helix-basic leucine zipper (HLH-bZIP) transcription factor that dimerizes with a second HLH-bZIP protein, MAX, to generate an E-box (CACGTG) binding protein (148). *c-myc* also interacts with a variety of chromatin components and transcription factors to modify the expression of its targets, numbering as many as ten percent or more of genes (149). Although the majority of these are up-regulated, multiple MYC repressible targets have also been identified. MYC targets, not surprisingly considering their abundance, are involved with many critical processes including proliferation, growth, development (*c-myc* knockout mice die during early embryogenesis), differentiation, apoptosis, and include metabolic enzymes, structural proteins, intra- and extracellular signaling molecules, transcription and chromatin regulatory proteins, as well as many of the

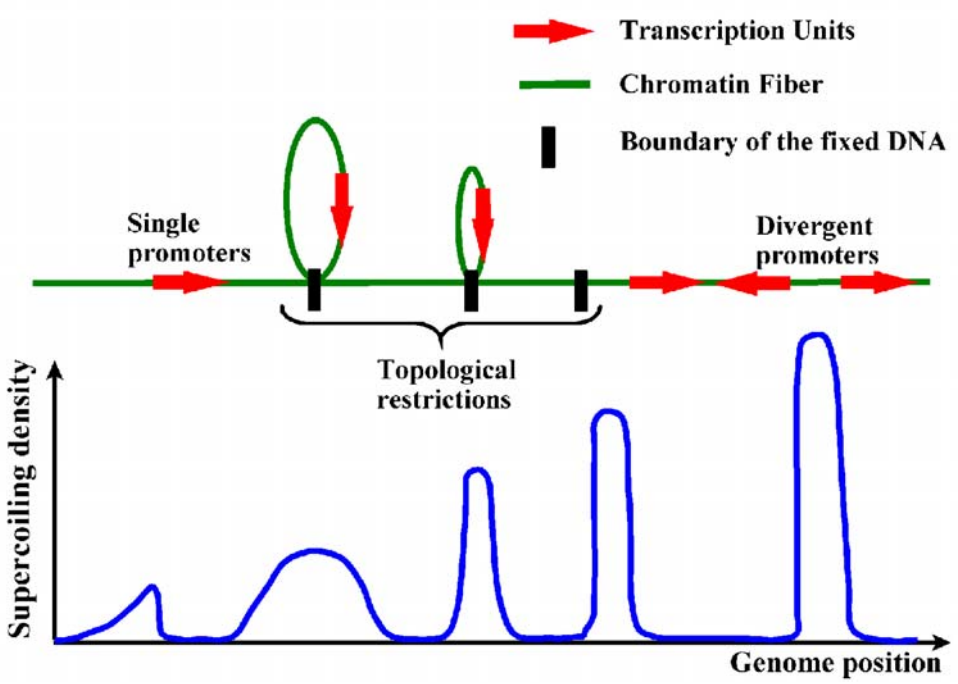

Figure 3. Influence of the chromatin structure and promoter arrangement (upper panel) on the amplitude and the transmission of transcription-generated dynamic supercoiling (lower panel). Note that the pattern of supercoiling distribution is also dependent on the rate of transcription, competition between different non-B-DNA structures and available topoisomerase activity.

molecules required for protein synthesis including the rRNAs transcribed by RNA polymerases I and III. Just as MYC targets include representatives of virtually all cellular processes and pathways, so the transcription of *c-myc* itself is regulated by a plethora of intra- and extra-cellular signals conducted to the *c-myc* gene via a multitude of transcription factors and chromatin components. *c-myc* expression is abnormal in most human cancers, becoming deregulated by chromosomal translocations, viral insertions, point mutations, deletions and insertions. Though slight changes in MYC abundance have profound phenotypic effects at the levels of both individual cells and whole animals, *c-myc* mRNA and protein levels are low enough in most cells so that stochastic fluctuations in expression levels would be anticipated. Special mechanisms have evolved to suppress wide swings and cell-to-cell variability of MYC expression (150).

For precise regulation of low-level basal *c-myc* transcription, stability is built into the basal promoter; this same stability must be overcome when *c-myc* is induced in response to activating signals. In response to serum, the shape and duration of the elicited pulse of *c-myc* transcription is managed moment-to-moment by a real-time feedback system (83). Although end-product feedback is employed for many metabolic and regulatory systems, the delay imposed by the transcription, splicing, RNA processing, RNA transport, translation, dimerization with MAX, nuclear import of the newly synthesized protein, protein modification, binding and action at target sites (and this whole cycle may need to be repeated if target proteins of MYC other than MYC itself contribute to the feedback regulation of *c-myc* transcription) is slow relative to the half-lives of *c-myc* mRNA and protein (20-30 minutes each). Fluctuations in the small number of *c-myc* mRNA molecules present in most normal cells coupled with induction to variable levels, would seem to be inevitable in this situation. Tight control demands regulatory adjustments to output on the same time scale as disturbances in the system. What parameters might be monitored to measure promoter output in real-time?

If transcribing RNA polymerases generate and transmit dynamic supercoiling forces through the double helix to SIDD sites, torque-induced conformational changes would reflect the intensity of ongoing transcription. In this situation DNA itself would play the role of a molecular speedometer cable. The transmission of the torsional stress to susceptible sites would be sensitive to a variety of parameters (Figure 3) (151-155). 1) Distance—in an open system damped by friction, the superhelical density would be expected to decay linearly between the torque source and the open end (or site of removal). 2) Chromatin-- how torsional stress is dynamically propagated through chromatin is not known, but some features of this process are predictable based upon known principles of chromatin structure. If the trajectory of the double helix upon the surface of nucleosomes *in vivo* is accurately depicted by the known co-structures of core nucleosomes with DNA, then any excess of stable or dynamic supercoils must be accommodated primarily within the linker regions. The degree to which the nucleosome-fixed DNA can absorb torsional stress is limited by the strength of the histone-DNA interactions. The fact that

the B-DNA upon nucleosomes is already slightly overwound, would seem to indicate little ability of the static structure to accommodate underwinding. Though the dynamic release of a nucleosome would facilitate diffusion of supercoils along a DNA segment, re-engagement of the nucleosome would re-fix the 1.05 negative supercoils associated with each octamer (156). Therefore the net result is that superhelical stress would be propagated in a saltatory fashion between linker regions with DNA-wrapped nucleosomes rotated en bloc either by physical rotation or reassociation of the nucleosome in a different helical phase. Moreover, because frictional resistance to rotation increases as a higher power of radius, the thickening of the DNA fiber promoted by packaging into chromatin would also extend and prolong the transmission of torsional stress. The modification of histone tails would be expected to influence capacity of nucleosomes to absorb and transmit superhelical stress. Whether by engaging the linker at the DNA entry or exit sites of the nucleosome or by engaging nearby nucleosomes or other chromatin components, it is likely that modifications, especially those that eliminate (acetylation) or fix (tri-methylation) positive charges, will alter the elastic properties of a chromatin fiber and its ability to transmit torsional stress. 3) The architecture of DNA bends and loops—the much greater friction and hindrance to rotation about the helical axis caused by DNA bends and loops would also impede the transmission and removal of torsional stress whether in naked DNA or in chromatin. The magnitude of this effect would be extremely sensitive to the size, position and arrangement of the chromatin in the loop. 4) The arrangement and activity of other genetic elements, especially promoters: Bi-directional or closely nested divergent promoters is common feature of the human genome (85, 86). The degree of mutual reinforcement between the negative supercoils generated at each start site would be sensitive to the distance separating these sites and to the intensity of transcription initiated at each promoter. Other arrangements of promoters would also modify the level of torsional stress residing within regulatory DNA sequences. For example, elements downstream of transcription units would experience duplex stabilizing, positive superhelical stress emanating from an approaching elongation complex. However once an elongation complex traverses a downstream element, in a very short window, this element switches from positive to negative superhelical stress. This switch has been speculated to couple ADAR1's Z-DNA binding and RNA editing functions (142, 144, 146). 5) Placement and fixation of topological boundaries—a fixed topological boundary would constrain all the torsional stress generated within that topological domain. Unless removed, this strain would rise rapidly to levels that would modify ongoing genetic processes and that could directly modify DNA conformation. 6) The deployment of topoisomerases—the placement of topoisomerases relative to promoters and to topological boundaries has the capacity to control the levels of torsional stress within a segment of chromatin. Within a static domain, topoisomerase action would be expected eventually to remove all supercoils. However, *in vitro* evidence indicates that dynamically, supercoils and topoisomerase may co-occupy a DNA segment and that even linear DNA behaves as if it were supercoiled while it is being actively cranked (53, 157). Although definitive measurement of the distribution and extent of supercoiling forces within and around active genes *in vivo* has not been achieved, some experiments support their existence. Because of the torque exerted by negatively supercoiled DNA, intercalating agents at subsaturating levels have an energetic preference to insert between bases in underwound (negatively supercoiled) as opposed to relaxed DNA. Therefore treatment with psoralen followed by exposure to ultraviolet light to generate psoralen mediated inter-strand cross-links, DNA extraction and denaturing electrophoresis enables the separation of psoralen cross-linked sequences from non-cross linked DNA. Indeed several such studies have revealed enhanced psoralen cross-linking in transcriptionally active regions and have inferred the existence of transcriptionallly generated supercoils. These studies could not determine whether such supercoiling is simply a waste-product of transcription or whether the torsional stress influences gene activity. In *Drosophila*, UV-cross-linking of biotinylated psoralen to polytene chromosomes has been used to directly visualize torsionally strained DNA (158).

One study showed that CUP1-promoter driven transcription in yeast was definitively not influenced by supercoiling (159). This study used the induction *in vivo* of a restriction enzyme to linearize an episome to relieve torsional stress. While these results likely preclude a global, generic roll for static torsional stress in transcription, if supercoiling promotes alternative DNA structures that in turn selectively bind particular positive or negative effectors of gene expression, then the outcome of these experiments would be dependent on the selection of the gene to be studied and its particular array and arrangement of cis-elements and their associated transfactors. In yeast chromosomes, where the genes are closely packed and the dynamic supercoils from one gene would surely run into the other and interfere with expression unless removed; selective pressure would likely limit the use of topological regulation. In yeast topoisomerase redundancy (or compensation) prevents the accumulation of static supercoils (160, 161). In contrast, in mammals large intergenic segments may buffer those genes that are not regulated by DNA topology from the torsional stress generated at their distant neighbors.

The partition between alternative structures and conformations associated with torsional stress are not only dependent on the free energy differences between different states, but this distribution is also dependent on the kinetics of the confromational change relative to the rate of transmission and the persistence of the torsional stress. Binding of structure specific factors could modify the outcome and the kinetics of structural isomerization by stabilizing the initial, intermediate, or final states in the reaction pathway. A striking example of this is the binding of FIR to FUSE that is licensed by a pulse of supercoiling. Although at the outset, FIR is unable to engage relaxed duplex DNAs, once bound to transiently stressed FUSE, this interaction survives subsequent relaxation for a prolonged period. This sort of asymmetric reaction pathway (hysteresis) is indicative of a kinetic barrier to complex formation. FIR contacts DNA at hyphenated points across an extended segment of melted DNA. Unless

destabilized, it would seem unlikely that all the requisite contact sites would be exposed for simultaneous recognition. However, once bound, the protein-DNA interaction is enduring (83).

So the pattern of transmission of torsional stress is customizable (and hence selectable) according to the peculiarities of each gene. Therefore the conformational responses of DNA to applied supercoiling forces are also situational. Any accounting of the biological and pathological diversity associated alternative DNA structures will be incomplete unless considered in the context of *in vivo* supercoiling.

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