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Z-DNA, an active element in the genome

Guliang Wang, Karen M. Vasquez

Department of Carcinogenesis, University of Texas M.D. Anderson Cancer Center, Science Park-Research Division, 1808 Park Road 1-C, Smithville, TX 78957

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1. ABSTRACT

Z-DNA is a left-handed helical form of DNA in which the double helix winds to the left in a zigzag pattern. DNA containing alternating purine and pyrimidine repeat tracts have the potential to adopt this non-B structure *in vivo* under physiological conditions, particularly in actively transcribed regions of the genome. Z-DNA is thought to play a role in the regulation of gene expression; Z-DNA is also thought to be involved in DNA processing events and/or genetic instability. For example, Z-DNA-forming sequences have the potential to enhance the frequencies of recombination, deletion, and translocation events in cellular systems. Although the biological function(s) of Z-DNA and related Z-DNA-binding proteins are not fully understood, accumulating experimental and clinical evidence support the idea that this non-B DNA conformation is involved in several important biological processes and may provide a target for the prevention and treatment of some human diseases. In this review, we discuss the properties of Z-DNA, proteins that are known to bind specifically to Z-DNA, and potential biological functions of this non-canonical DNA structure.

2. INTRODUCTION: LEFT-HANDED Z-DNA CONFORMATION

DNA is capable of adopting many different conformations in addition to the canonical Watson-Crick B-DNA. To date, at least 10 different types of non-B DNA conformations have been identified, and it is likely that other non-B conformations will be discovered.

Z-DNA is a left-handed double helix named after its zigzag arrangement of the sugar-phosphate backbone of the DNA molecule, and was first described in the late 1970s (1). In fact, the first single-crystal X-ray structure of a DNA duplex exhibited a left-handed structure. In a Z-DNA structure, the purines are inverted and in the *syn* conformation, while the pyrimidines remain in the *anti* conformation such that the pyrimidine nucleotide must rotate to maintain the Watson-Crick base-pairing (2). Such an alternating *syn-anti* conformation drives the backbone into a zigzag shape. The major groove essentially disappears in a Z-DNA conformation, while the minor groove remains and maintains its narrow character, corresponding to the B-DNA structure. At the B-Z junction

region, at least one base pair is thought to be disrupted, and the two bases extrude from the DNA duplex on both sides (3). The Z-DNA structure is also more compact relative to B-DNA, with an average of one additional base-pair per turn. How such a significant conformational change from B-DNA to Z-DNA occurs at the atomic level is still a question that remains unanswered. Several models have been proposed to explain the B-to-Z transition [(4), and references therein], including base-pair opening before rotation (1); a series of non-disruptive transfiguration steps for transition (2, 5); an A-type or a stretched intermediate conformation with (4) or without (6) disruption of Watson-Crick base-pairing; and a "zipper" model of sequentially extruding base-pairs from the duplex, permitting their free rotation, consistent with the crystallographic structure of a B-Z DNA junction (3).

Because purines adopt the *syn* conformation more readily than pyrimidines, Z-DNA conformation occurs most readily in alternating purine/pyrimidine d(PuPy) or d(PyPu) repeat sequences, especially alternating guanosine and cytosine residues (7, 8), followed by d(TG)n repeats, and d(TA)n repeats, though TA repeats tend to adopt a hairpin structure more readily than a Z-DNA structure. GT repeats are the most frequent simple repeating element found in the human genome with estimates of this repeat accounting for more than 0.25% of the total genome (9). Additionally, sequences other than alternating purine-pyrimidine regions, such as d(GGGC)n, have also been shown to adopt a Z-DNA conformation (10, 11).

3. BIOLOGICAL ACTIVITIES THAT FACILITATE Z-DNA FORMATION *IN VIVO*

Pohl *et al.* (1972) observed that, under high salt conditions (*e.g.*, 4M NaCl), a double-stranded poly d(GC) oligomer underwent a near complete inversion of the ultraviolet circular dichroism spectrum; this inversion was subsequently explained by X-ray diffraction studies that revealed that the oligomer had adopted a Z-DNA conformation (12). Because Z-DNA is a higher energy conformation than B-DNA, the existence of this structure under physiological (low-salt) conditions has been debated. However, it is now recognized that Z-DNA is not limited to formation *in vitro*, as there are numerous lines of evidence demonstrating the existence of Z-DNA structures *in vivo* [for reviews, see (13, 14)].

Z-DNA formation *in vivo* is a dynamic process. DNA sequences adopt a canonical B-form structure the majority of the time with non-B structures occasionally arising as transient intermediates (14). There are a variety of biological or pathological activities that may serve as an impetus for DNA to adopt non-B conformations. For example, when potential Z-DNA-forming sequences are negatively supercoiled, the energy from the torsional stress can convert the B-DNA into a Z-DNA conformation, since each change of a right-hand turn to a left-hand turn would be associated with the removal of 1.8 helical turns of supercoiling. Jaworski *et al* (1991) showed that the level of Z-DNA formation on a CG repeat was consistent with the level of supercoiling *in vivo*; the Z-DNA conformation was increased in a topoisomerase I mutant, and was decreased in gyrase mutated *E. coli* strains (15). In addition, Z-DNA was formed at a GT(32) sequence, detected by a Z-DNA antibody, only in a negatively supercoiled plasmid, but not in a relaxed plasmid (16). This finding suggested that Z-DNA could exist *in vivo* under physiological conditions because chromosomal DNA of both eukaryotes and prokaryotes is generally negatively supercoiled. Negative supercoiling can be induced and maintained on a topologically closed region of DNA, *e.g.*, closed circle such as double-stranded viral DNA or plasmid DNA, prokaryotic chromosomal DNA, and segments of long linear molecules of eukaryotic chromosomal DNA bound with proteins on both ends. In eukaryotes, negative supercoiling is generated during the wrapping of DNA around the histones in a left-handed fashion, and positive stress is released by topoisomerases at histone-free DNA regions to ensure DNA is not torsionally strained (17). Unwrapping of the nucleosome releases the energy associated with DNA negative supercoiling that was absorbed during the DNA nucleosomal wrapping process. During transcription, RNA polymerase opens duplex DNA, but does not rotate when moving forward on a DNA template, resulting in tight positive supercoiling in front (released by topoisomerases) and negative supercoiling behind the progressing polymerase. In addition, C^5 methylation at cytosine residues, which is very common in the human genome, can significantly stabilize the Z-DNA conformation under physiological conditions (9). Methylation can increase the helical pitch of DNA (18), lower the free energy of Z-DNA formation, and promote helix unwinding at B-Z junctions (19). A methyl group on cytosine in B-DNA is protruded into the aqueous solvent phase from the major groove of the B-DNA helix and interacts with water molecules, thereby destabilizing the B-DNA structure (20). Although the methyl groups bring only a slight modification in the structure of Z-DNA, they are in close van der Waal's contacts with both the imidazole ring of guanine and the carbon atoms of the sugar, forming a hydrophobic patch on the surface of the molecule to stabilize the Z-DNA conformation (20). When methylated, a linear GC repeat can form Z-DNA with three orders of magnitude less magnesium than required by unmethlyated DNA (21). Polyamines such as spermine and spermidine, which are commonly found in eukaryotic cells, also stimulate or stabilize the Z-DNA structure (22-25).

4. Z-DNA-BINDING PROTEINS

Because the Z-DNA conformation has been postulated to function as a potential *cis* element, identification of proteins that bind in a structure-specific fashion to Z-DNA could help elucidate the biological role(s) of Z-DNA *in vivo*. In the past decades, a substantial number of Z-DNA-binding proteins have been identified in vaccinia virus (26-29), *Escherichia coli* (30-32), *Saccharomyces cerevisiae* (33), and in Drosophila (34, 35), wheat germ (36), zebrafish (37), chicken (38), rat (39), bovine (40), and human cell nuclei (30, 39, 41, 42). Although the functions of most of these Z-DNA-binding proteins have not been completely established, existing evidence suggest that they play important roles in gene

expression, DNA recombination, RNA editing, viral pathogenicity, and tumor development (14). Double-stranded RNA adenosine deaminase 1 (ADAR1), E3L and DLM-1 are among those Z-DNA-binding proteins studied in the most detail and are discussed below. The Z-DNA-binding domain, Z-alpha, from these three proteins demonstrates high sequence similarity, adopting a winged-helix conformation and binding to Z-DNA in a structure-specific manner (43).

4.1. Double-stranded RNA adenosine deaminase 1, ADAR1

ADAR1 is member of a deaminase family that edits double-stranded mRNA, and its expression is interferon (IFN) inducible. By converting adenines into inosines, ADAR1 is an important molecule for generating RNA and protein diversity. It has two Z-DNA-binding motifs, Z-alpha and Z-beta. Z-alpha belongs to the family of winged-helix proteins and is able to bind specifically to the left-handed Z-DNA conformation with a K_d of ~4 nM (44). An interaction between Z-alpha (ADAR1) and supercoiled plasmid DNA with GC(13) or GC(2)CGC(10) inserts was observed by atomic force microscopy (29). Furthermore, Z-alpha (ADAR1) binding was found to induce the B-to-Z transition when the supercoiling level on the plasmids was not at a level to form a Z-DNA structure in the absence of protein (29). Binding of ADAR1 to a Z-DNA structure can remarkably enhance its A-to-I editing efficiency *in vitro* (45). It is proposed that a Z-DNA structure induced by a moving RNA polymerase at an appropriate sequence is able to recruit ADAR1 protein at the transcription site behind the transcription machinery to edit the newly synthesized RNA (44, 46). In addition to RNA editing, a study using a reporter gene under the control of a minimal promoter with a Z-DNA-forming sequence nearby demonstrated that Z-DNA and ADAR1 interaction increased promoter activity (44). When a Z-alpha (ADAR-1) is expressed in the cell, a substantial increase in transcription of the reporter gene was also observed. This result suggests that Z-DNA formation in the promoter region induced or stabilized by a Z-DNA-binding protein, or the Z-alpha (ADAR-1) protein recruited to the promoter itself is involved in transcription regulation (44).

4.2. Vaccinia virus (VV) E3L protein

E3L is an IFN resistance gene encoded by vaccinia virus (VV). The N-terminal domain of the E3L protein has sequence similarity to the Z-alpha region found in ADAR1 and DLM-1, but has lower Z-DNA-binding affinity compared with its homologues, due to residue Y48 in the Z-alpha domain that adopts a different side chain conformation which requires rearrangement for binding to Z-DNA (28). Both Z-alpha and C-terminal double-stranded RNA binding domains are required for VV replication in a wide range of host cells (47), and for pathogenesis in a mouse model (48). Vaccinia viruses containing an N-terminal deletion (including the Z-DNA-binding domain) of E3L are attenuated for neuropathogenesis and fail to infect the lungs or brain after intra-nasal installation of C57BL/6 mice. Researchers are considering using the recombinant virus as a vaccine for protecting against smallpox because of decreased neurovirulence (49). VV retains pathogenicity when the E3L Z-DNA-binding domains are replaced by a similar Z-DNA-binding domain from ADAR1 or DLM-1 (50). Recently, the E3L protein (or the Z-alpha domain alone) was found to increase reporter gene expression in HeLa cells by 5- to 10-fold when the minimal promoter contained IL-6, NF-AT, or p53 elements upstream of the TATA box; it even increased the expression by 2-fold from a basic promoter containing only a TATA box, but showed no effects on a reporter gene either lacking a TATA box or containing a CREB, AP-1, or an NF kappa B element upstream of the TATA box. Further, E3L protein, its Z-alpha domain, or chimeric proteins where the Z-alpha domain was replaced with Z-alpha domain from ADAR1 or DLM-1, significantly protected HeLa cells from hygromycin-B-induced apoptosis in a dose- and time-dependent manner (26). If a deletion or a point mutation was introduced into the Z-alpha region, the protection effect disappeared, indicating that Z-DNA-binding is essential for these activities (26).

4.3. DLM-1

DLM-1 is also known as Z-DNA-binding protein 1 (ZBP1). It was identified as a Z-DNA-binding protein by searching sequence databases for homologues of the Z-alpha region of ADAR1 (43). DLM-1is a tumor associated gene, predominantly expressed in lymphatic tissues and is highly up-regulated in the peritoneal lining of mice bearing mouse ovarian ascites tumor (51). DLM-1 expression is also induced by IFN-gamma or lipopolysaccharide in mouse peritoneal macrophages, so it is speculated to be involved in host responses against cellular stresses, including tumorigenesis and viral infection (51). In addition to the Z-alpha domain, DLM-1 also has a conserved domain homologous to the second Z-DNA-binding domain of ADAR1, Z-beta (39). Both domains are capable of binding Z-DNA, and their binding activities are comparable to that of the Z-alpha domain from ADAR1 (52). Comparison of Z-DNA binding by DLM-1 and ADAR1 reveals a common structure-specific recognition core within the binding domain (43). A potential role of DLM-1, and particularly a role of its Z-DNA binding ability in tumor development and stress response remains unclear.

Z-DNA-binding proteins share common structural characteristics; for example, peptides with alternating lysines (such as KGKGKGK) can bind to Z-DNA specifically (53), and such sequences can be found in many proteins. In many cases, the Z-DNA-binding domains from different proteins can substitute for each other, as discussed above. Further, wild-type E3L protein is a potent inhibitor of ADAR1 deaminase enzymatic activity, probably by competing with the binding site on Z-DNA. Disruption of the Z-DNA-binding domain of E3L by double substitutions of two highly conserved residues abolishes its antagonistic activity (27). These observations provide strong evidence that the interaction of the Z-DNA-binding domain and the Z-DNA structure is crucial for carrying out the biological function of these proteins.

5. Z-DNA AND HUMAN DISEASES

5.1. Z-DNA and chromosomal translocation breakpoints in blood cancers

A common genetic alteration of blood-cell cancers (leukemia, lymphoma and myeloma) is

chromosomal translocation. Immunoglobulin and T-cell receptor genes are the most frequent receptor loci in related translocations because these genes have active promoters in related blood cells and DNA double-strand breaks (DSBs) are naturally generated in these genes during V(D)J recombination in the production of rearranged active antibody or antigen-receptor genes (54, 55). Interestingly, in many cases breakage hotspots found in related oncogenes have homologies to neither V(D)J sequences nor recombinase recognition sequences, indicating that they are not the result of V(D)J recombination (56). Instead, they often map to regions that are capable of forming Z-DNA or other non-B-DNA structures. The t(12;21)(p13;q22) translocation, fusing the ETV6 and AML1 genes, is the most frequent chromosomal translocation associated with pediatric B-cell precursor acute lymphoblastic leukemia. Several breakpoints and a stable insertion polymorphism are found very near an alternating purine-pyrimidine Z-DNA-forming sequence in the ETV6 gene (57). Boehm *et al.* have examined the breakpoints of three different lymphoid tumor-specific translocations where related genes on both chromosomes are not actively transcribed. In all three translocations studied, a B cell tumor-specific translocation t(11;14)(q13;q32) and two T cell-associated translocations, t(1l;14)(p13;q11) and t(7;10)(q35;q24), stretches of alternating purine-pyrimidine residues were found within a short distance of the breakpoint region: an 800 bp Z-DNA-forming sequence was found close to the 11q13 chromosome breakpoint; chromosomal breakage hotspots in four independent tumors with a t(11;14)(p13;q11) translocation contain an adjacent potential Z-DNA region of 62 bp in length at 11p13; and a 32 bp long purine--pyrimidine tract was found at a 10q24 translocation site (58). Foroni *et al.* also reported a similar observation of a Z-DNA-forming sequence at a breakage site on chromosome 10q24 (59). *BCL-2, c-MYC* and *SCL* are among those most frequently translocated genes in leukemia, lymphoma, and myeloma; and the recurring breakpoints in these genes have been sequenced in many studies. The t(14;18)(q32;q21) rearrangement juxtaposing the *BCL-2* gene to the immunoglobulin heavy chain gene locus, resulting in constitutive overexpression of *BCL-2* is the hallmark of low-grade non-Hodgkin's lymphomas. Ninety percent of follicular B cell lymphomas carry this translocation (60). The majority of t(14;18) chromosome translocations occur at the 3' region of the *BCL-2* gene where the V(D)J recombination signal sequences were found, indicating the involvement of V(D)J recombinase complex in generating the breaks (61). The 5' breakage hotspot of the *BCL-2* gene (62-68) is the most commonly recurring breakage site in chronic lymphocytic leukemia. Interestingly, multiple Z-DNA-forming elements were found surrounding this common breakpoint and Z-DNA structures were shown to exist *in vivo* using Z-DNA specific antibodies (69, 70). The human *c-MYC* gene is frequently juxtaposed through chromosomal translocation to one of the immunoglobulin loci on chromosomes 2, 14, or 22 (71-73) and the t(8;14)(q24;q32) translocations are found in ~90 % of Burkitt's lymphomas (74). Single-stranded elements in the *c-MYC* gene (75), an indicator for non-B DNA structure conformation, overlap the breakpoint-hotspot at the 5' flanking region near the P1 promoter (76, 77) and the boundary of exon 1 and intron 1 (78). Interestingly, multiple alternating purine-pyrimidine Z-DNA-forming regions have been found near the *c-MYC* P1 promoter and the 3' downstream region (79, 80) near these breakpoints. The *SCL* gene (also known as *TCL5* or *tal-1*) dysregulation can result from joining *SCL* to the *TCRD* or *TCRB* gene in t(1;14) or t(1;7) translocations, and are the most common chromosomal abnormality associated with T-cell acute lymphoblastic leukemia (ALL), found in ~30% of patients with childhood T-cell ALL (81). A translocation breakpoint cluster region and a 90-kb deletion region are found 5' to the coding region of the *SCL* gene. The V(D)J recombinase complex is involved in some *SCL* translocations (82). However, alternating purine and pyrimidine Z-DNA-forming sequences are frequently found in close proximity to the breakpoint when V(D)J recombinase is not involved. In the (1;7) translocation, a 38 bp stretch of alternating GT was found 43 bp 5' of the translocation breakpoint on chromosome region lp32 (83). In a t(1;3) translocation, a 94 bp tract of alternating purine and pyrimidine sequences were found at the breakpoints on chromosome 3 (82). These findings suggest a close relationship between Z-DNA structures and chromosomal breakage and translocation. Recently, studies from our laboratory showed that both Z-DNA and H-DNA induce DNA DSBs in mammalian cells (84, 85), providing further support for this concept.

5.2. Z-DNA and other non-malignant diseases

In addition to chromosomal translocations involved in blood cancers, there is some evidence to suggest that Z-DNA may also play a role in other human diseases. For example, an increased amount of Z-DNA has been identified in DNA from the hippocampus of Alzheimer's patients but not in normal brain DNA, although the role of Z-DNA in disease development has not yet been established (86). The natural resistance associated macrophage protein 1 (*NRAMP1*) gene (also named *SLC11A1*) plays an important role in determining human susceptibility to autoimmune (rheumatoid arthritis, juvenile rheumatoid arthritis, type 1 diabetes, Crohn's disease) and infectious (tuberculosis, leprosy) diseases. A potential Z-DNA-forming sequence in the promoter of the *NRAMP1* gene is involved in its transcriptional regulation, and a polymorphism in this Z-DNA-forming dinucleotide repeat contributes directly to disease susceptibility [(87-89), and references therein]. Z-DNA might also be responsible for metal ion-induced carcinogenesis (90, 91). It was proposed that binding of metal ions, such as nickel ions, might induce and/or stabilize Z-DNA structures (92, 93), and may also react specifically with DNA in the Z conformation resulting in selective base damage which may eventually lead to carcinogenesis (discussed later in this review).

6. Z-DNA AND TRANSCRIPTION - CAUSE AND EFFECT

Computer-based programs searching for sequences capable of forming Z-DNA structures reveal a strong bias for their location in transcription initiation sites in 137 human genes studied (94). Thus, many studies have been performed to determine the role of Z-DNA in the regulation of gene expression [(95) and references therein].

A tight correlation between Z-DNA formation and transcriptional activity has been observed (96, 97); transcription can induce Z-DNA conformation and Z-DNA structure can regulate transcription activity.

6.1. Transcription and induction of the Z-DNA conformation

Because RNA polymerase does not rotate when moving on a DNA template, transcription results in negative supercoiling behind the polymerase, thus facilitating Z-DNA conformation at permissive regions. Antibodies against Z-DNA bind preferentially to the sites that are actively transcribed (98-100), suggesting that transcription can induce Z-DNA conformation, or the sequences undergoing transcription are unwrapped from the nucleosome and therefore may be more accessible for antibody binding. *In situ* assays in nuclei isolated from Allium cepa L. root meristems to detect active RNA polymerases resulted in images that were comparable to those obtained from *in situ* immunodetection of Z-DNA; and inhibitors to RNA polymerase decreased or diminished the Z-DNA staining (101). In permeabilized nuclei from mammalian U937 cells, prepared to ensure the biotin-labeled Z-DNA antibody was able to diffuse into the nuclei while maintaining transcription activity, staining of streptavidin in the nuclei indicated that binding of anti-Z-DNA antibodies was associated with the level of transcription (102). Other examples include experiments demonstrating that three fragments of the *c-MYC* gene near the promoter were found to be bound with Z-DNA antibody only when the *c-MYC* gene was actively transcribed (79, 103).

6.2. Z-DNA and regulation of transcriptional activity

Although transcription seems to enhance or stabilize Z-DNA formation, Z-DNA may either enhance or repress transcriptional activity. Fragments containing Z-DNA-forming sequences in the rat nucleolin (*Ncl*) gene, [(CA)(10)(CG)(8)] in the promoter region, and [AC(GC)(5)CCGT(CG)(2)] in the first intron, were isolated and enriched by a Z-DNA antibody in metabolically active permeabilized nuclei; deletion of [(CA)(10)(CG)(8)] in the promoter region increased the promoter activity by ~50% (104). However, alternating purine-pyrimidine tracts have also been shown to result in a strong inhibitory effect to the expression of the tRNAPro gene from Caenorhabditis elegans when placed in the flanking regions of the gene or in the promoter (105). Thus, Z-DNA may regulate gene expression *via* different mechanisms, perhaps dependent on its location and stability (see below).

6.2.1. Z-DNA and chromosomal remodeling in transcription regulation

DNA bound to the histone octamer retains the B form and is energetically stabilized by DNA-histone interactions (106). Unwrapping DNA from the nucleosome generates negative supercoiling and can induce Z-DNA structures at permissive sequences. On the other hand, forming a Z-DNA structure can affect nucleosome packaging. In one study, histone octamers were reconstituted on plasmids carrying GC repeats; when plasmids were supercoiled, the CG repeat was much more susceptible to micrococcal nuclease digestion compared with control B-DNA sequences on supercoiled plasmids. In addition, in relaxed plasmids, the CG repeat was not susceptible to nuclease digestion, suggesting that DNA in the Z form is not efficiently incorporated within core particles, and that a transition from the B to the Z form *in vivo* might result in a significantly altered local placement of nucleosomes (107, 108). The colony-stimulating factor 1 (*CSF1*) gene contains TG repeats in the promoter which are assembled in nucleosomes when *CSF1* is not transcribed. Upon activation of *CSF1* expression, the chromatin-remodeling enzyme BRG1 disrups this nucleosome, resulting in chromatin remodeling, but not in transcription-initiated Z-DNA formation at the GT repeat in mammalian SW-13 cells (109). Once in the Z form, the promoter region could not be packaged as a nucleosome and was therefore in an open conformation, resulting in increased levels of transcription (109). More recently, a similar result was found in *Saccharomyces cerevisiae*; a CG(9) repeat was found to stimulate gene activity by forming a Z-DNA structure when placed approximately three helical turns (28 bp) or fewer upstream of the *CYC1* TATA box. A nucleosome-scanning assay revealed that the CG(9) repeat sequence in the Z conformation blocks nucleosome formation, producing transcriptionally favorable locations for the TATA box (110).

6.2.2. Z-DNA; local and global supercoiling levels in transcription regulation

It is now well known that promoter activity is regulated by supercoiling levels. RNA polymerase requires transient melting of the DNA duplex at the transcription initiation start site, which can be facilitated by local negative supercoiling stress (111, 112). Negative supercoiling of DNA also facilitates an interaction between transcription factors and DNA, such as transcription factor II D and the fibroin gene promoter, demonstrated in a *in vitro* system using partially purified components from HeLa cells (113). As another example, increasing DNA supercoiling by high osmolarity in growth media resulted in approximately 8-fold higher levels of invasive gene *invA* transcription in *Salmonella typhimurium* than that in lower osmolarity conditions; reducing the level of DNA supercoiling by gyrase inhibitors also reduced expression of *invA* (114). The fibroin gene and adenovirus 2 major late promoter were fully transcribed when templates were supercoiled. Supercoiling can also repress transcription initiation; expression of the histidine biosynthetic operon of Salmonella typhimurium is increased by relaxation of supercoiled DNA (115, 116).

A left-handed helical turn of Z-DNA formed at a promoter region can relieve 1.8 helical turns of right-handed negative superhelical twisting (117), therefore Z-DNA can have substantial effects on gene expression rates *via* changing the local DNA conformation. The relative propensities to undergo transition is ordered according to the energies required to drive Z-DNA structure and the energy relieved by the relaxation of supercoiling. For example, a highly negatively supercoiled ($\Delta LK = -50$) $ilvP_G$ promoter has 60- to 70-fold higher activity than its activity on a relaxed template and shifting the supercoiling

level from ΔLK= -12 to -26 increases promoter activity by five-fold. However, a Z-DNA structure at a promoter region can absorb the supercoiling stress that is required for promoter activation, thereby suppressing the promoter activity (118). A similar effect was demonstrated when Z-DNA was located at a distance from the promoter (119). Increasing the supercoiling level at the *gyrA* (DNA gyrase, subunit A) promoter reduced its activity and a Z-DNA insert actually suppressed the inhibition of supercoiling at the promoter (118). A GT repeat in the *CSF1* promoter, which can form Z-DNA *in vitro*, is required for SWI/SNF-like BAF dependent activation in SW-13 cells. Other Z-DNA-forming sequences can substitute for the GT repeats and maintain the promoter activity, suggesting that it is the structure but not the primary sequence that is required for promoter activity (120). Further, binding of the BAF complex increased the Z-DNA conformation at GT repeats, as indicated by an increased cleavage by a Z-DNA-specific restriction enzyme *in vivo* after BAF binding to the promoter region (120). The authors proposed that the mammalian BAF complex is capable of transiently unwrapping DNA from the nucleosome, resulting in negative supercoiling and a Z-DNA structure at the histone-free GT repeat (109, 120). Moreover, once a Z-DNA structure is adopted, it presents a barrier for the DNA to be re-wrapped into nucleosomes and this region is likely to stay open longer for transcription initiation.

6.2.3. Transcription machinery stalling at Z-DNA structures

Since the conformation of left-handed Z-DNA is dramatically different from B-DNA, it's not surprising that templates containing Z-DNA are not ideal for RNA polymerases if not removed by helicases. *E. coli* RNA polymerase (RNAP) was shown to be blocked at the boundary of a negatively supercoiled CG(16) sequence in the Z-DNA conformation *in vitro*, while when that sequence was relaxed and in the B-form, it had no effect on transcription (121). A high error rate for wheat germ RNAPII was observed when CG repeats served as a template (122). We have shown, in collaboration with the Hanawalt group, that shorter transcripts of discrete lengths were obtained in an *in vitro* transcription assay using Z-DNA as a template with T7 RNAP. This result was more pronounced after multiple rounds of transcription, while transcription of the control insert produced full-length transcripts, indicating that the presence of Z-DNA affected the processivity of T7 RNAP (unpublished data).

6.2.4. Z-DNA protein binding in transcription regulation

Z-DNA formation can also influence transcriptional activity by interfering with the binding of required transcription factors. If a sequence containing a response element adopted a Z-DNA conformation, then it might not be able to recruit the related transcription factors. For example, when a transcription factor E2F binding site GCGCGAAA has some GC or GT repeats incorporated in an upstream adjacent area, it might adopt a Z-DNA conformation *in vivo* preventing E2F binding. Moreover, Z-DNA-binding proteins (see above) might bind to the target DNA sequence with a higher affinity than the transcription factors, interfering with their binding and/or function (123).

7. Z-DNA-INDUCED GENETIC INSTABILITY

Genetic instability is a hallmark of several human diseases including cancer and neurological disorders. There is now substantial evidence indicating that non-B DNA structures can induce genetic instability (124-126). Expansions of triplet repeat elements are known causative factors in the diseases such as Fragile X syndrome, Huntington's, Friedreich's ataxia, and myotonic dystrophy (127, 128). Certain "fragile sites" or "hotspot" regions of the genome that are prone to undergo DNA breaks, translocations, deletions and amplifications (129) are also associated with DNA structure. Zhang and Rowley's review (130) recently summarized the colocalization of *in vivo* topoisomerase II cleavage sites and DNase I hypersensitive sites with genomic breakpoint hotspots in several chromosomal translocations in leukemia. Notably, DNA structure, rather than DNA primary sequence is responsible for both topoisomerase II and DNase I cleavage *in vivo* (131-133). Z-DNA is one of the most frequent sequence elements occurring at the hotspots. DNase I cleaves at the single-stranded DNA region at B-Z junctions, and topoisomerase II prefers to bind and cleave at Z-DNA-forming sequences (134-136), see below.

7.1. Z-DNA-induced deletion of repeat units in bacteria

It is well known that plasmids carrying Z-DNA-forming sequences are very difficult to maintain in *E. coli*, suggesting that these structure are unstable in bacteria. When plasmids containing Z-DNA-forming CG repeats, ranging from 6-29 units, are replicated in bacteria, deletions occur at a frequency substantially greater than in plasmids without the CG repeats (137, 138). Freund *et al.* measured the stablity of CG repeats in a *lacZ* mutation-reporter plasmid in bacteria using both "lacZ+ to lacZ-" and "lacZ- to lacZ+" assays, and found that the Z-DNA-forming CG repeats (ranging from 6-13 units) underwent deletions. For CG(12) and CG(13), ~10% of the total mutants propagated in JM103 cells contained small deleltion/insertion events of 1-2 repeat units; the remaining 90% of mutants underwent larger deletions within the repeat sequence. The authors proposed that the small deletions/insertions (of 1-2 units) within the repeats were most likely due to slippage events during replication, based on the following observations: 1) the (GT)12, and (AT)12 plasmids showed a similar frequency of 1-2 repeat unit deletions/insertions as CG(12); and 2) the frequency of small deletion/insertion events in a mutL-deficient strain was increased ~4-fold compared to the wild-type strain, suggesting that these mutants are product of slippage events left uncorrected due to deficient mismatch repair. However, the larger deletions found within the GC repeats were found to be mutL independent. This process appears to be specific to the Z-DNA structure and not to a possible hairpin structure because an AT(12) repeat, which is as susceptible as CG(12) to form a hairpin structure, but does not adopt a Z-DNA conformation, showed a much lower frequency of large deletions compared to the CG(12) repeat sequence (139). It is possible that the Z-DNA structure, particularly when bound and stabilized by proteins, results in DNA polymerase pausing, giving rise to larger or multiple rounds of deletions until the CG repeat is too short to form a stable Z-

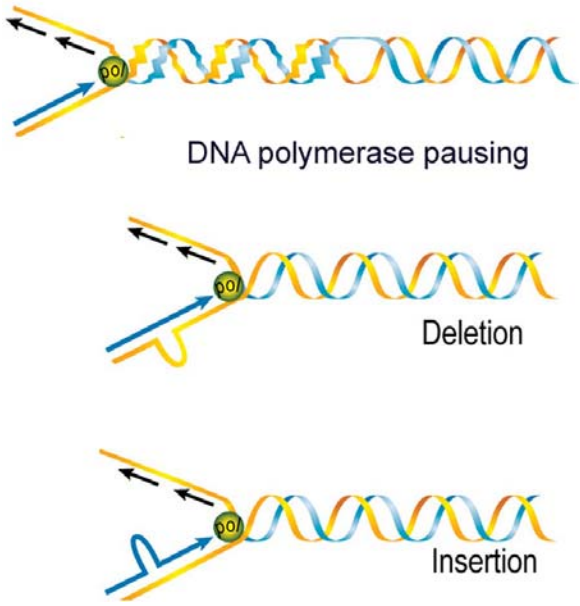

Figure 1. Z-DNA-induced deletion in bacteria. DNA replication fork pausing at a Z-DNA conformation can result in an increased risk of nascent-template strand disassociation and misalignment. A loop formed in the template strand results in deletion of repeats, while a loop in the nascent strand results in insertion. The same events could occur repeatedly until the CG repeat element is too short to adopt Z-DNA.

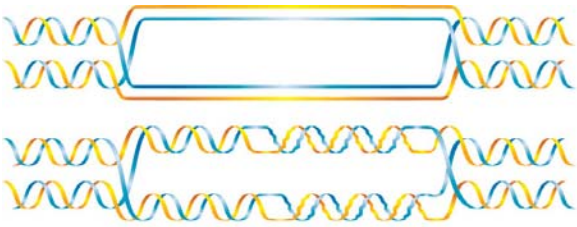

Figure 2. Possible roles of Z-DNA in recombination. Two molecules form a Holliday junction at the homologous region. If both strands are intact, when the strands wrap around each other to form right-handed B-DNA, a region of similar length on the other end must be in a left-handed conformation. A Z-DNA-forming sequence can greatly facilitate and stabilize this type of Holliday junction (adapted with permission from reference 124).

DNA conformation (Figure 1). Studies from our laboratory provided additional evidence to support this idea: the sequences UY1 (GCGCGCGCGTGTGCGCGCGTGCGCGCGC) and RW1009 (CGCGCGCGCGTATATACGCGCGCG), which contain GT or AT inserts to interrupt the direct CG repeats and reduce the slippage events, showed significantly lower frequencies of small repeat unit deletions in bacteria DH5 alpha, compared with CG(14) repeats (84).

The mechanism of Z-DNA-induced genetic instability is not clear. Although RecA is required for CG repeat-induced intermolecular recombination (to form dimers or multimers) (137), the deletion events at CG repeats do not require the recombination proteins, RecA or RecBC (137). However, Z-DNA-containing plasmids are much more stable in bacterial SURE® cells (Stratagene) which are defective in genes encoding the nuclease, SbcC, the recombinational proteins, RecB and RecJ, and repair proteins, UmuC and UvrC, indicating that recombination and/or repair activities may be involved in the Z-DNA-induced genetic instability in *E. coli*.

7.2. Genetic recombination and Z-DNA-induced genetic instability

A Z-DNA motif GT(30) repeat cloned into a suercoiled plasmid stimulates RecA-indepedent recombination between GT repeats in bacteria at a much higher frequency than background levels (140). There are numerous studies demonstrating that Z-DNA-forming sequences correlate with recombination hotspots in eukaryotic cells (141, 142). The presence of a 34 bp GT purine-pyrimidine tract was shown to enhance reciprocal meiotic recombination in yeast (143). A hotspot of 1,000 bp in the major histocompatibility complex (MHC) in mice, where several copies of long GT repeats are found, may account for 2% of recombination on the entire chromosome (144, 145). RecA binds to Z-DNA with a faster on rate than to B-DNA, though is not necessary for Z-DNA-induced recombination (146). The difference is a kinetic effect rather than an effect at equilibrium; it binds to both Z- and B-DNA with a stoichiometry of 1 monomer/4 base pairs (146). However, other studies suggest that the high affinity binding of RecA to GC repeats can be attributed to the sequence rather than the structure (32, 147). Proteins purified from human tumor cells using Z-DNA affinity chromatography have an activity to catalyze a recombination strand-transfer reaction similar to that of the RecA and Rec1 proteins (148).

Topologically, when an intact single-stranded DNA molecule binds to its intact homologous strand and folds into a right-handed helix during homology recognition, the adjacent region must adopt a left-handed conformation. A Z-DNA-forming sequence can thus facilitate recombination by stabilizing this intermediate. Conditions that impair Z-DNA conformation such as addition of anti-Z-DNA antibodies or alteration of the super-helical density of the DNA inhibits strand pairing, suggesting that transient formation of Z-DNA is required for Recl-mediated pairing of duplex molecules during recombination reactions (149) (Figure 2). Alternatively, the DSBs induced by Z-DNA (discussed below) might also lead to recombination by providing a DSB intermediate. The recombination induced by Z-DNA is not necessarily restricted to essentially identical loci. An unequal sister chromatid exchange can occur, resulting in genetic instability (150).

7.3. Z-DNA and DNA double-strand breaks in mammalian cells

A recent study from our laboratory demonstrated that a CG(14) Z-DNA-forming sequence induces high levels of genetic instability in both bacterial and mammalian cells. In mammalian cells the CG(14) repeats

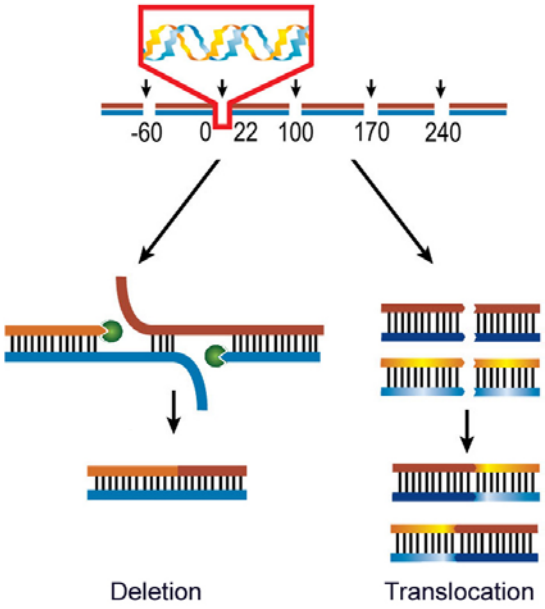

Figure 3. Z-DNA structure-induced genetic instability in mammalian cells. Z-DNA formed *in vivo,* shown in the red box, is recognized and cleaved by as yet undefined enzymes in mammalian cells, resulting in DSBs (chromosomal breakages) surrounding the structure. The hotspots of DSBs induced by a CG(14) repeat in mammalian cells (84) are shown in the schematic diagram. The error-prone repair at DSBs results in large-scale deletions, translocations and rearrangements.

induce DSBs surrounding the Z-DNA structure resulting in large-scale deletions of the entire repeat element and the flanking mutation-reporter gene. The Z-DNA-induced DSBs are dispersed over a 400 bp region, consistent with chromosomal breakpoints in human diseases such as leukemias and lymphomas (151, 152). Moreover, CG(14)-induced small deletions in the repeats require replication, during which misalignment may lead to deletions and/or expansions. Interestingly, the large-scale deletions, which are the predominant event in mammalian cells (~95% of the mutants) but are very rare in bacteria (<5% of the mutants), are replication-independent. Therefore, we postulate that the large-scale deletions induced by CG(14) are likely initiated by repair processing cleavages surrounding the Z-DNA structure (84), see Figure 3.

The enzyme(s) responsible for the recognition and cleavage of Z-DNA in mammalian cells remains unclear. Potential candidates include DNA topoisomerases, which generate transient DNA DSBs during DNA replication. However conditions that increase the frequency of these DNA breaks can trigger mutagenic events or apoptosis in yeast (134). A cluster of strong binding and cleavage sites for DNA topoisomerase II are located near Human Immunodeficiency Virus (HIV) integration sites in the human genome. Interestingly, these sequences consist of a complex repeating element, and Z-DNA motifs (135). Drosophila melanogaster DNA topoisomerase II binds to and relaxes negatively supercoiled DNA containing Z-DNA-forming sequences (136). In addition, purified chicken topoisomerase II has cleavage activity on both DNA strands in a 54 bp alternating purine-pyrimidine region from the human beta-globin gene, which is also a hotspot for recombination and mutation *in vivo*. The activity of this enzyme on alternating purine-pyrimidine sequences appears to be proportional to the length of the repeating sequence (153). These findings suggest a role for DNA topoisomerase II in Z-DNA-induced DSBs and genetic instability.

7.4. Accumulation of DNA damage in the Z-conformation

Different types of DNA damaging agents can cause non-random lesions along the DNA. The type of DNA motifs to be preferentially attacked depends upon the chemical or physical nature of the assaulting agent and the DNA base composition and structure. In a normal B-DNA structure, the base-pairing between strands is protected within the helix. However, the guanosine nucleotides are in a *syn* position in a Z-DNA conformation, with the purine base located over the sugar without protection, thus possibly being more accessible to DNA damaging factors (154). The abnormal positioning or absence of nucleosomes can also change the sensitivity of the DNA to genotoxic agents. UV irradiation has been shown to induce significant chromatin fragmentation at repetitive sequences, particularly at GT repeats (a Z-DNA-forming sequence) and long stretches of homopurine/homopyrimidine regions (potential H-DNA-forming sequences) (155). The guanine sites in the Z-DNA structure are more sensitive, and the cytosine sites less sensitive to ionizing radiation, leading to a characteristic signature of the Z-form DNA (156). Hyperreactivity to certain agents has also been observed at the B-Z junction (157). Although guanine N^7 lies on the surface of the major groove in B-DNA, access of Ni^{2+} and Co^{2+} to guanine is apparently inhibited by the steric constraints. However, the solvent accessibility of guanine N^7 increases greatly upon formation of Z-DNA, therefore Ni^{2+} selectively induces oxidative damage on guanine in the Z-DNA, but not in the B-DNA conformation (158). In addition, damage occurring to the DNA in a Z-DNA conformation is likely more resistant to processing by DNA repair enzymes. For example, bases damaged by alkylation such as N^7-methylguanine and O^6-methylguanine, are removed by a DNA glycosylase, and O^6-methylguanine-DNA methyltransferase, respectively, in B-DNA but are not efficiently repaired by these enzymes when present in Z-DNA (159, 160). Thus, it is possible that endogenous (oxidative) DNA damage accumulates at Z-DNA regions contributing to their induction of genetic instability.

8. METHODS TO MODULATE Z-DNA CONFORMATION *IN VIVO*

Since the Z-DNA structure plays important roles on DNA metabolic processes *in vivo*, researchers are attempting to find or create small molecules that can interfere with Z-DNA specifically, to either enhance or inhibit the biological functions in which Z-DNA participates.

Transition metal ions are able to promote the Z-DNA conformation at alternating purine and pyrimidine sequences *in vitro* (161, 162). Monovalent alkaline cations drive a B to Z transition in high salt (~2.3 M for Na^+ and 4.7 M for Cs^+). Mg^{2+}, Ca^{2+}, and Ba^{2+} cations induce Z-DNA structure formation at poly[d(G-C)]. The divalent transition metal ions Zn^{2+}, Cu^{2+}, Mn^{2+}, Co^{2+}, and Ni^{2+} tend to coordinate to guanosine N^7, which is more accessible in the Z-DNA than in the B-DNA structure, and drive the B to Z transition at low concentrations [(163), and references therein]. Aluminum, which has been found to be increased in the serum samples of Fragile X syndrome patients (1.8×10^{-6} M vs. 0.53×10^{-6} M in unaffected individuals), can induce Z-DNA structure formation at a CCG(12) repeat localized at the 5' end of the non-coding regions of the Fragile X mental retardation-1 (FMR1) gene *in vitro*. Once formed, the structure was quite stable even after the total removal of aluminum from the reaction (164). On the other hand, chloride anions inhibit the Ni^{2+} promoted Z-DNA conformation *in vitro* very effectively by modulating the interaction of nickel ions with DNA (165). The anticancer drug (+)-daunorubicin binds selectively to right-handed B-DNA. It can also allosterically convert Z-DNA-forming sequences to a B-DNA form; however, its synthesized enantiomer, (-)-daunorubicin (WP900), binds selectively to left-handed DNA, and can convert B-DNA to a Z-DNA form at sequences with the potential to adopt Z-DNA (166). The naturally occurring polyamines putrescine, spermidine, and spermine can induce Z-DNA structures *in vitro* (23). Drugs that inhibit the production of polyamines have therefore been tested for the treatment of human autoimmune disease where a Z-DNA structure is involved. The survival rate of female MRL-*lpr/lpr* mice was increased by 29% by treatment with Difluoromethylornithine (DFMO), a drug that inactivates ornithine decarboxylase and thus reduces the production of polyamines. This effect was suggested to be the result of the inhibitory action of DFMO on cell proliferation and the prevention of DNA from forming an immunogenic Z-DNA conformation (167).

In addition to molecules that can change DNA structure directly, compounds that bind to the Z-DNA-binding proteins and interfere with DNA-protein interaction might also be useful in modulating Z-DNA-related metabolism. Through structure-based screening of a chemical database, Kim *et al.* (168) discovered small molecules that could bind to a Z-DNA-binding protein and inhibit its interaction with Z-DNA. According to the X-ray crystal structure of Z-alpha domain of human ADAR1 protein bound to Z-DNA hexamer d(CGCGCG)2, three non-charged small compounds containing several hydrogen bond acceptor atoms out of a total of 78,425 compounds in a chemical database were found to have the ability to interact with Z-DNA-binding protein and inhibit the interaction of the protein with the Z-DNA, identified by inhibition of Z-DNA-binding in gel-shift assays. Although further work is required to increase the affinity and specificity of these compounds to Z-DNA, such efforts provide the potential of development of a new class of therapeutic agents based on interacting with Z-DNA for the treatment of related human diseases.

9. CONCLUSION

The discovery that Z-DNA and other non-B DNA structures are actively involved in many biological and pathological processes has changed our thinking about how the physical structure of genes (DNA) impacts on disease development. The exact functional/mechanistic role(s) of Z-DNA *in vivo* is not yet completely understood (due in part, to experimental difficulties posed by the transient nature of the Z-DNA structure), but certainly substantial evidence exists to support its involvement in these processes. Clearly, additional research is needed to address the developmentally relevant temporal and tissue specific influences of the Z-DNA structure. Identification of the complete set of trans-acting factors that interact with the Z-DNA and their disease relevance is also of paramount importance. Exciting progress is currently being made in several laboratories to discover ways to modulate the conformation of DNA in particular regions of the genome as well as to influence the binding of proteins that interact with non-canonical DNA structures as strategies to prevent or treat human disease.

An insightful commentary on the status of the field has been contributed by Rich and Zhang (14), and is well summarized in this excerpt from their review that the research is still in its infancy and there is still a “long road to biological function”. The potential of exploiting modulation of DNA structure for therapeutic benefit should eventually result in a new field of disease prevention and therapy.

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Abbreviations: ADAR1: Double-stranded RNA adenosine deaminase 1; IFN: interferon; VV: Vaccinia virus; DSBs: double-strand breaks; ALL: acute lymphoblastic leukemia; NRAMP1: natural resistance associated macrophage protein 1; CSF1: colony-stimulating factor 1; RNAP: RNA polymerase; HIV: Human Immunodeficiency Virus

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Send correspondence to: Karen M. Vasquez, Ph.D. University of Texas M.D. Anderson Cancer Center, Department of Carcinogenesis, 1808 Park Road 1-C, P.O. Box 389, Smithville, TX 78957, Tel: 512-237-9324, Fax: 512-237-2475, E-Mail: kvasquez@mdanderson.org

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