been anticipated. Instead of all the residues having bases organized in the anti-conformation, this molecule every other base rotated around the glycosyl bonds so that the bases alternated in anti- and syn-conformations along the chain. Also, there was a zigzag arrangement of the backbone of the molecule (hence, the name Z-DNA) that looked different from the smooth continuous coil seen in models of B-DNA (Fig. 1). Instead of having a helix with a major and minor groove, the base pairs were set off to the side, away from the axis, and only one groove was found that was analogous to the minor groove of B-DNA. The bases that form the major groove in B-DNA were reorganized in Z-DNA to form the convex outer surface.

The general response to this unusual structure was amazement, coupled with scepticism. However, after a brief period of excitement, the biological community largely ignored Z-DNA, as it did not seem to be required to explain anything. Recent research on this alternative conformation of the double helix has began to show that Z-DNA has important biological functions.

Conformation and stability

The relationship between Z-DNA and the more familiar B-DNA was indicated by the earlier work of Pohl and Jovin, who showed that the ultraviolet circular dichroism of poly(dG-dC) was nearly inverted in 4 M sodium chloride solution. The suspicion that this was the result of a conversion from B-DNA to Z-DNA was confirmed by examining the Raman spectra of these solutions and the Z-DNA crystals. The conversion of B-DNA to Z-DNA was associated with a ‘翻’over of the base pairs so that they had an upside down orientation relative to that of B-DNA. This flipping over resulted both in the production of a syn-conformation in every other base and a change in the deoxyribose–ring pucker in alternate bases. The net result of this reorganization was that the phosphate groups were closer together in Z-DNA than in B-DNA. Hence, under standard cellular conditions, the electrostatic repulsion of these charged phosphate groups would push the molecule into the B-DNA conformation. In the presence of a high-salt solution, the electrostatic repulsion of the phosphate residues is vastly decreased, and Z-DNA becomes the stable conformation.

Several studies quickly showed that chemical modification, including cytosine methylation and many other cations, such as spermine and spermidine, would stabilize the Z conformation. It emerged that the lowest energy level ground state of DNA in a physiological solution was B-DNA, and that the Z conformation was a higher energy state. Because purines can form the syn-conformation without an energy penalty, it became apparent that the specific sequence of the base pairs was important in determining the energy that was required to change B-DNA to Z-DNA. The sequences that most readily converted had alterations of purines and pyrimidines, especially alternations of C and G. This change also occurred easily with alternations of CA on one strand and TG on the other. However, many other sequences were shown to be capable of forming Z-DNA.

This discovery stimulated a burst of research from chemists who were interested in studying DNA conformational changes. Although the ionic conditions that were suitable for stabilizing Z-DNA in most experiments were different from those present in a cell, spermine, spermidine and cytosine methylation, which also stabilize Z-DNA, are found in vivo. Furthermore, the discovery that negative supercoiling would stabilize Z-DNA indicated biological involvement. Negative supercoiling requires energy and tends to unwind B-DNA. For example, in a plasmid with three negative supercoils, if one turn of the DNA helix changed from right-handed to left-handed, two negative supercoils would disappear and the energy of negative supercoiling would then stabilize a small segment of Z-DNA (Fig. 2). Supercoiling was known to be a part of biological systems, which indicated a connection between this alternative conformation and biological phenomena. Early experiments showed that the negative supercoiling of plasmids in prokaryotes would stabilize Z-DNA.

Many in vitro experiments were carried out to determine the energy that was required for a supercoiled plasmid with a particular sequence to flip from the B form to the Z form. The energetics of these conformations was studied for several different sequences. This ultimately led Ho et al. to devise a computer program that made it possible to calculate the relative energy required to flip any sequence from the B form to the Z form.

Workers in several laboratories determined crystal and solution structures of DNA sequences in the Z conformation. These provided a great deal of detailed information about the Z conformation and, at the same time, many chemists discovered ways in which the Z conformation could be stabilized relative to the B conformation. Nonetheless, work on the biology of Z-DNA progressed slowly. By the mid-1980s, after several years of research, nothing definitive had emerged about Z-DNA function. During this period, although some notable studies supported a functional role for Z-DNA in transcription (see later), others showed that the influence of Z-DNA on transcription was dependent on the gene that was examined, which increased scepticism and decreased enthusiasm for the study of the biological role of Z-DNA. Many people felt that Z-DNA was a non-functional
conformational phenomenon and this attitude became relatively widespread. So, although chemists continued to find Z-DNA interesting, by the end of the 1980s the biology of Z-DNA was not receiving attention from researchers, and its study had largely disappeared, except in the Rich laboratory.

Transcription and Z-DNA

In fact, the groundwork towards showing a biological role for Z-DNA came from immunological research that was carried out years before the downturn of interest in this area. Unlike B-DNA, Z-DNA is highly antigenic; both polyclonal and monoclonal antibodies can be raised to Z-DNA molecules. The characterization of these antibodies led to the discovery that Z-DNA-specific antibodies are found in human autoimmune diseases, especially in systemic lupus erythematosus. Antibodies to Z-DNA also provided a useful tool for characterizing chromosome organization. They bound specifically to the interband regions of the Drosophila polytene chromosomes, and this binding was particularly strong in the puff regions, which are the sites of enhanced transcriptional activity. Others found the same staining patterns in unfixed polytene chromosomes. However, some studies have shown antibody binding outside these regions.

Further studies in protozoa also indicated a link to transcription. Ciliated protozoa have two nuclei; the macronucleus, which is the site of transcription, and the micronucleus, which contains DNA that is involved in sexual reproduction. Anti-Z-DNA antibodies stained the macronucleus of the ciliated protozoan Stylonychia, but not its micronucleus.

A real breakthrough came with the work of Liu and Wang, in 1987, on the interaction of the RNA polymerase complex with the DNA duplex during transcription. They suggested that the complex does not rotate around the helical DNA, but instead plows straight through. Because the ends of the DNA molecule are fixed, the DNA behind the moving polymerase is unwound and subjected to negative torsional strain, which is known to stabilize Z-DNA. In front of the moving polymerase, positive torsional strain develops.

Further evidence came from the work of Ho and colleagues, who first studied the distribution of sequences that favoured Z-DNA formation in 137 human genes. They found a high concentration of these sequences near the transcription start sites. In more recent experiments, Ho scanned human chromosome 22 and estimated that ~80% of its genes have sequences that favour Z-DNA formation near the transcription start site. (P. S. Ho, personal communication). As these were not present in many pseudogenes, Z-DNA-forming sequences near the transcription start site might have a functional role.

To study the association between Z-DNA and transcription more directly, Rich collaborated with Wittig and colleagues to use a technique developed by Cook at Oxford University. Mammalian cells were encapsulated in agarose microbeads and mild detergent treatment lysed the cytoplasmic membrane and permeabilized the nuclear membrane, but left the nucleus otherwise intact. The resulting ‘entraped’ nuclei replicated DNA at nearly the in vivo rate and were able to carry out transcription. Using biotinylated monoclonal antibodies against Z-DNA, the level of Z-DNA was determined in these nuclei, and was shown to be regulated by torsional strain. Moreover, an increase in the transcriptional activity of the embedded nuclei resulted in a parallel increase in the amount of bound Z-DNA. Using a short ultraviolet (UV) laser pulse for protein–DNA cross linking, they linked the biotinylated anti-Z-DNA antibodies to DNA. This made it possible to isolate DNA restriction fragments that were bound to the antibody and probe them for specific nucleotide sequences. They found that three regions near the promoters of the C-MYC gene formed Z-DNA when C-MYC was expressed (Fig. 3), and the Z-DNA-forming nucleotides were identified. However, these regions quickly reverted to B-DNA if C-MYC transcription was switched off.

Figure 3 | Transcription stabilizes Z-DNA.

Metabolically active permeabilized nuclei were used to show that C-MYC transcription is associated with the formation of Z-DNA in three restriction fragments, which are labelled Z1–Z3 (numbers above the boxes show the size of the fragments). These are all found near the C-MYC promoters, which are labelled P0–P3 (REF. 26). When transcription is turned off, the continued action of topoisomerases relaxes the Z-DNA, which disappears entirely after ~15 minutes. Therefore, Z-DNA is formed transiently in association with transcription.

Timeline | From Z-DNA structure to function

The first single-crystal structure of a DNA fragment unexpectedly reveals a left-handed double helix, named as Z-DNA.

Liu and Wang recognize that negative supercoiling is produced behind a moving RNA polymerase during transcription.

The transcription of human C-MYC is correlated with Z-DNA formation near its promoter in permeabilized nuclei.

It is shown that the Z-DNA binding domain of ADAR1 binds Z-DNA as well as Z-DNA.
the constitutively expressed actin control retained its Z-DNA at all times. This showed a correlation between transcription and Z-DNA conformation, which has also been found in other genes28.

So, the negative torsional strain induced by the movement of RNA polymerases stabilizes Z-DNA formation near the transcription start site. Although topoisomerases tried to relax the DNA, the continued movement of RNA polymerases generated more negative torsional strain than the topoisomerases could relax. However, on the cessation of transcription, topoisomerase action rapidly converted the DNA back to the B conformation. So, Z-DNA was seen as a metastable conformation that formed and disappeared depending on physiological activities.

How is Z-DNA formation initiated in transcription? One answer was afforded by the recent work of Liu et al. who studied the chromatin remodelling system SWI/SNF29. This complex of proteins helps to turn on genes by unwrapping DNA from nucleosomes. Liu et al. studied the human colony stimulating factor 1 gene (CSF1), which is turned on by this system. Unwinding the nucleosome leaves the DNA negatively supercoiled as a result of the way in which it is wrapped around the nucleosome. Liu et al. discovered that the released DNA was in the Z conformation. It had been known for several years that Z-DNA could not form nucleosomes28. So, the nucleosome cannot reform and the site is kept open, which allows the accumulation of other transcription factors and the initiation of transcription by RNA polymerase. They showed that transcription was triggered by Z-DNA formation. Given the prevalence of sequences that favour Z-DNA formation near transcription sites, it is possible that this mechanism is widespread22.

Proteins that bind Z-DNA

Identifying binding proteins. If Z-DNA were to have biological functions, it seemed highly likely that there would be a class of proteins that would bind specifically to it. Several attempts were made to isolate such proteins, using columns and other techniques. Early attempts met with limited success but did result in the important and serendipitous discovery that is described in Box 1.

The problem with Z-DNA-binding proteins was devising a method that would make it possible to isolate proteins that bound selectively to Z-DNA with high affinity. Herbert developed a powerful technique for identifying Z-DNA-binding proteins with the exclusion of proteins that could bind to B-DNA30. The method used a gel-shift assay with radioactive-labelled chemically stabilized Z-DNA in the presence of a ~20,000-fold excess of B-DNA and single-stranded DNA. This technique detected proteins that bound specifically and tightly to Z-DNA and led to the isolation, in 1995, of a Z-DNA-binding nuclear-RNA-editing enzyme22 called double-stranded RNA adenosine deaminase (ADAR1).

ADAR1 acts on double-stranded segments that are formed in pre-mRNA, binding to the duplex and selectively deaminating adenosine to yield inosine. Ribosomes interpret inosine as guanine, so it can alter the amino-acid sequence of a DNA-encoded protein. A typical substrate of this enzyme is an RNA duplex in which an exon is paired with a region of an intron. The deaminase edits several pre-mRNAs, including a glutamate receptor that is expressed in the central nervous system (CNS)33. The receptor is an ion channel, and a glutamine residue near the centre of the channel is changed through editing to arginine; its positive charge restricts the entry of calcium ions, a change that is essential for CNS function. Another substrate is the serotonin receptor33. In all of these cases, the functional properties of the edited protein (with the amino-acid alteration) are detectably different from those of the unedited protein. The editing enzyme is found in all metazoans and acts to increase the functional diversity of proteins that are transcribed from a given locus.

Box 1 | Discovery of self-assembling peptides from the study of zuotin

Zhang developed a simple gel-retardation assay using stabilized methylated Z-DNA ([d(SmC)G]n) to purify the first naturally occurring left-handed Z-DNA-binding protein. A yeast protein called zuotin was found to bind Z-DNA in the presence of a 400-fold molar excess of right-handed B-DNA46. Zuotin had an interesting repetitive sequence motif — AEAEAKKEAEAKK — which has been extensively developed by Zhang to create a class of β-sheet peptides that are self-complementary as a result of the presence of both positive and negative side chains on one side of the β-sheet and hydrophobic side chains on the other44. This serendipitous discovery of a self-complementary peptide inspired Zhang to design several new members of this peptide class, which form 3-dimensional (3D) nanofibre scaffolds that can be used in 3D cell cultures46–50. The four self-complementary peptides shown here — RAD16-I, RAD16-II, EAK16-I and EAK16-II (the segment from yeast zuotin) — form stable β-sheet structures in water and undergo spontaneous assembly to form nanofibre scaffolds. These nanofibre scaffolds hold large volumes of water (>99.5% water content). Tissue cells can be embedded in a 3D nanofibre scaffold in which they can establish molecular gradients that often mimic the in vivo environment.

Other related self-assembling peptide systems have also been designed, which range from ‘molecular switch’ peptides that undergo marked conformational changes50 to ‘molecular carpet’ peptides for surface engineering to peptide nanotubes and nanovesicles51,52, all of which originated with the Z-DNA-binding zuotin discovery.
A Z-DNA-binding domain

Proteolytic dissection of the editing enzyme ADAR1 made it possible to isolate a domain from the N-terminus, called ZαADAR1, which contains all the Z-DNA-binding properties that are associated with the editing enzyme34. This domain was used by Kim et al. to create a conformationally specific restriction endonuclease that would only cut Z-DNA35,36.

Several experiments were carried out to illustrate the interaction of the ZαADAR1 domain with DNA in solution. If the dodecamer d(CG)6, was put in solution, it produced the typical circular dichroism spectrum of B-DNA. As ZαADAR1 was added to the physiological solution, the spectrum gradually changed, which reflected conversion to the Z form37. This showed that the Zα domain of ADAR1 was capable of stabilizing the dodecamer in the Z conformation, which was probably generated by Brownian motion that twisted the dodecamer fragment. After flipping into the Z-DNA conformation, the ZαADAR1 domain binds to the DNA and prevents it returning to the B conformation. In later experiments, a similar phenomenon was shown in a longer DNA molecule in which the d(CG)6 segment was inserted between two longer segments with sequences that would not easily form Z-DNA38. In such cases, the ZαADAR1 would bind to the central region, holding it in the Z conformation, whereas the flanking regions remained in the B conformation. Therefore, the binding energy was great enough to hold a small segment of DNA in the Z conformation and also provided enough energy to stabilize the two B–Z junctions.

Oh and Kim developed a yeast one-hybrid system to study Z-DNA-binding proteins in vivo39. They discovered that when ZαADAR1 has been fused to an activation domain binds to Z-DNA near a promoter, it enhances the transcription of the reporter gene. However, even without the activation domain, a level of transcriptional activation remains. These findings are consistent with the suggestion of Liu et al.40 that Z-DNA formation near the promoter would stimulate transcription.

Schwartz et al. discovered that the purified ZαADAR1 domain from ADAR1 could be crystallized with d(CG)6. The crystal structure, solved at a resolution of 2.1 Å (REF. 40), showed that the DNA was identical in form to that seen in the first crystal of Z-DNA41.

Box 2 | Z-RNA

The discovery of the structure of left-handed Z-DNA naturally led to the question of whether RNA could also form this conformation. In 1982, chemically modified oligoribonucleotides indicated that this was a possibility42, and further nuclear magnetic resonance, circular dichroism and absorption spectroscopy studies strongly indicated that Z-RNA could be formed in high-ionic-strength solutions43,44. More detailed structural information was obtained from X-ray crystallographic studies with chimeric hexamers that were made of alternating CG residues in which the two central CG residues were ribonucleotides, whereas the flanking pair of nucleotides were deoxyribonucleotides. These and other structural studies showed that the conformation of Z-RNA was similar to that of Z-DNA44,45. Z-RNA was also found to be immunogenic, and, although Z-RNA specific antibodies could be isolated46, some antibodies recognized both Z-RNA and Z-DNA. Staining experiments with Z-RNA-specific antibodies showed that the antibody bound to fixed protozoan cells that were visualized by immunofluorescence microscopy. The antibodies were mostly found in the cytoplast, which indicated that some cytoplasmic sequences in fixed cells exist as Z-RNA47. Cytoplasmic microinjection of anti-Z-RNA antibodies was found to inhibit cell multiplication48.

The experiments on Z-RNA were mostly carried out in the late 1980s, and since 1990 there have been no further publications. However, a possible physiological role for Z-RNA was suggested recently by Brown and Lowenhaupt et al., who found that the Z-DNA-binding domain of the editing enzyme double-stranded RNA adenosine deaminase (ADAR1) could bind to Z-RNA and Z-DNA49 with similar affinity. It had been known for some time that certain RNA viruses that replicate in the cytoplasm undergo considerable changes in sequence, which were probably the consequence of hyper-editing by ADAR1. Sequence analysis of the virus found in measles encephalitis showed that the RNA undergoes many edits: adenines are replaced by guanines, and uracils by cytosines. So, this virus has been extensively hyper-edited43,44. E3L is a small protein that is necessary for pathogenicity45. If vaccinia virus is given to a mouse, the mouse dies within one week. However, a virus that has a mutated or missing E3L is no longer pathogenic for the mouse, even though the virus can still reproduce in cell culture46. The protein has an N-terminal domain with a sequence that is characteristic of Z-DNA.
binding (Z_{E3L}) and a C-terminal domain that has a dsDNA-binding motif. In infected cells, E3L is exported to the nucleus where it accumulates.

**Viral pathogenicity**

To investigate the pathogenicity of the vaccinia virus in the mouse, and its relationship to the possible Z-DNA-binding activities of E3L, a collaboration was set up between the Rich laboratory and the Jacobs laboratory. Chimeric viruses were created in which the N-terminal domain of vaccinia E3L (Z_{E3L}) was removed. Z_{E3L} has sequence similarities to Zα_{DLM1} and Zα_{AD1}. Two chimeric viruses were created in which the two known Z-DNA-binding domains were substituted for Z_{E3L}. In carrying out these domain swaps, a little more than a dozen amino acids in the domain remained unchanged; these were the residues that were known to bind Z-DNA in the co-crystals. However, more than 50 other residues were changed. The chimeric viruses were as pathogenic as the wild type when the N-terminal residues of vaccinia E3L were deleted, the mice survived. ZE3L has sequence similarities to the Z-DNA binding protein in the nucleus of the infected cell. If such a molecule rescued mice that were infected with vaccinia virus, it might also be active in humans. This molecule could be developed to eliminate the harmful side-effects of vaccination. More importantly, the E3L protein of the closely related variola virus, which is the agent of smallpox, is almost identical to the vaccinia E3L. So, small molecule drugs that bind to E3L might make it possible to develop a therapy for smallpox.

This is the first example in which a Z-DNA-binding protein has been found to be involved in viral pathogenesis. If other viruses use a similar mechanism to downregulate the host response, then these proteins might be potential targets for anti-viral drugs.

**Conclusions**

The Z-DNA conformation has been difficult to study because it does not exist as a stable feature of the double helix. Instead, it is a transient structure that is occasionally induced by biological activity and then quickly disappears. The discovery and biological activity of Z-DNA-specific binding proteins point the way to a broader understanding of its biological roles. One active area of research will be the comparison of the occurrence of Z-DNA-forming sequences and Z-DNA-binding proteins between prokaryotes and eukaryotes; already, there are indications that sequences that form Z-DNA are less frequent in prokaryotes (P. S. Ho, personal communication). What we have seen so far is just the beginning, but it has provided insights that are likely to stimulate more research into this unusual left-handed version of the DNA double helix.

**Glossary**

- **ADAR1**: The editing enzyme double-stranded RNA adenosine deaminase, which converts adenosine to inosine in pre-mRNA. This enzyme has an N-terminal domain that binds tightly to Z-DNA.
- **ANTI- AND SYN-CONFORMATIONS**: Nucleic-acid bases can rotate about the glycosyl bond. The Watson–Crick hydrogen-bonding point away from the sugar in the anti-conformation (as in B-DNA), and have the opposite orientation in the syn-conformation. Purines can form the syn-conformation more easily than pyrimidines.
- **BROWNIAN MOTION**: The random kinetic thermal motion of molecules.
- **DNA FIBRE X-RAY DIFFRACTION ANALYSIS**: X-rays are scattered by electrons and if a molecule has regular periodicities, they will be detected by the diffraction pattern. In this technique, DNA molecules are orientated so that their long axes are parallel. Although the diffraction pattern can provide some information about the molecule, the conclusions are often tentative because the number of reflections is relatively small.
- **CIRCULAR DICHROISM**: This method measures the difference in absorption of right and left circularly polarized light as it passes through a solution containing molecules that absorb at that wavelength. The circular-dichroism spectrum is plotted as a function of wavelength.
- **POLYTWINE CHROMOSOME**: A chromosome that has duplicated many times and has remained laterally associated so that it is visible, as seen in Drosophila salivary glands.
- **RAMAN SPECTRUM**: Measures the vibrations of molecules that are usually influenced by the conformation of a molecule. This can be obtained from crystalline materials as well as materials in solution.
- **SINGLE-CRYSTAL X-RAY DIFFRACTION**: In this technique, a molecule is crystallized to produce many repetitions that are organized in a regular threedimensional array. This produces X-ray diffraction with a large number of reflections. Solution of this crystal structure can establish the conformation of the molecule because large amounts of redundant data are collected.
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Entrez
E3L

DLM1

zuotin

LocusLink
C-MYC

CSF1

OMIM
systemic lupus erythematosus

Shuguang Zhang's laboratory
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